Interactomics: Basics & Applications

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Lecture - 23
Applications of Protein Arrays in Identification of Autoantibody Signatures-II

Hello students, in the previous lecture I talked to you about using protein arrays how one could screen autoantibodies from the brain tumour patients. Idea is to give you the workflow what is involved of doing the actual biological experiments, actual clinical experiments where one could try to obtain the meaningful insight from these protein chips or different type of proteomic technologies.

So, in the last lecture, I essentially talked to you about the workflow involved in doing these experiments, and then I talked to you about one case studies on gliomas serum sample how one could probe that on the chip and try to find the potential biomarkers for autoantibodies screening.

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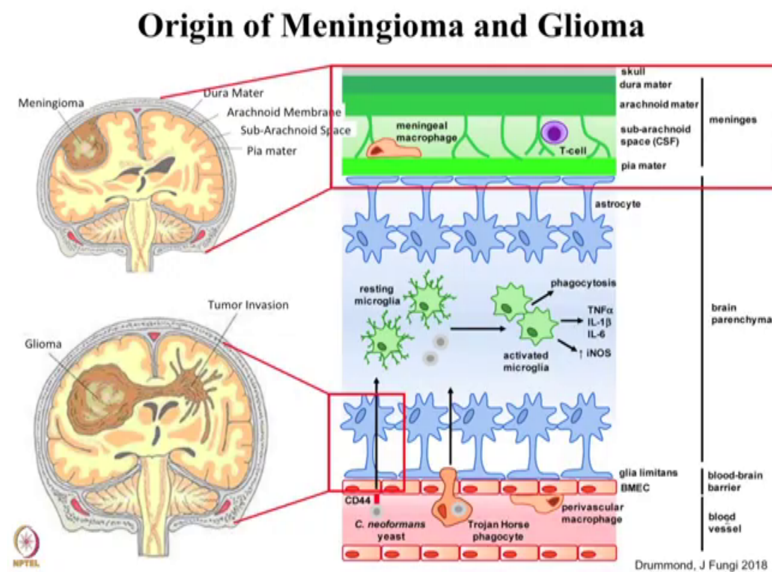

Let me continue bit more on that, because it is important for us to know that same platform could be used for variety of similar diseases, and also one could not only use the serum sample, but also other type of bio fluids. So, I am going to continue that application further, and talk to you about another disease right now which is meningioma.

Just to give you brief context to that the meningiomas, they derived from meninges of the brain which is you know the dura mater, arachnoid and pia mater, just go back little bit basics of your anatomy and think about the geology, and look at you know the brain structure. And you understand that you know while it looks like broadly they are all brain tumour, but they within the different regions of the brain these kinds of tumors can arise, and actually their aggressiveness and the way of treatment are very different depending on the location as well.

So, these tumors which are meningioma, these are most common most predominant type, almost 37 percent of among the all these CNS tumors, but other tumor which you can see the bottom panel which is a gliomas while they are or you know the not the highest numbers, but they are the most aggressive like the gliomas multiforme (Refer Time: 02:12), they are most aggressive tumor type and they have the origin differently which is from the glial cells

So, meningiomas in general they are slow growing, well-differentiated and demarcated, they are mostly benign, but of course, as the disease progresses towards the grade II length and three they might invade the brain and then you know one could have even the various malignant effect and potentially the death of the patient. But that is not so common as compared to if you think about the you know the glioma type of tumors which are fibrillary and diffused in nature, and they are very aggressive and very malignant, and they migrate and invade the entire normal brain tissues.

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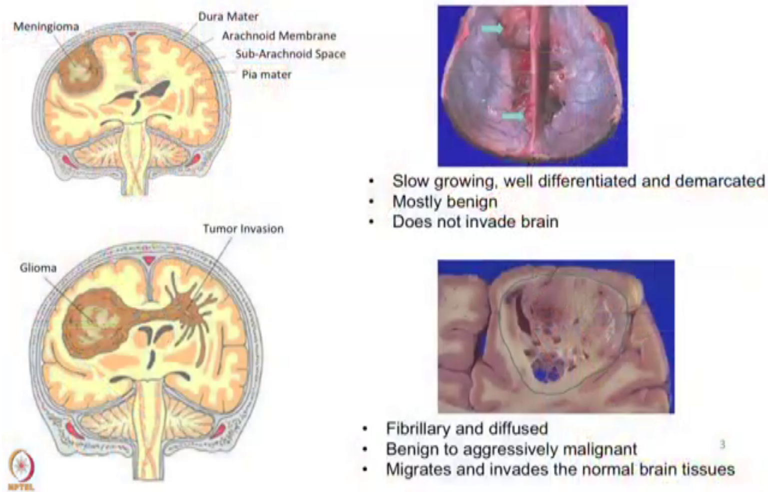

So, I hope you got some idea that you know or there are a different type of tumors. And when you are obtained the patient serum sample from different type of brain tumors, there is a curiosity that is the same type of antibodies are going to be seen or there are going to be different set of antibodies which are going to be showing the signal in response to glioma differently and meningioma differently.

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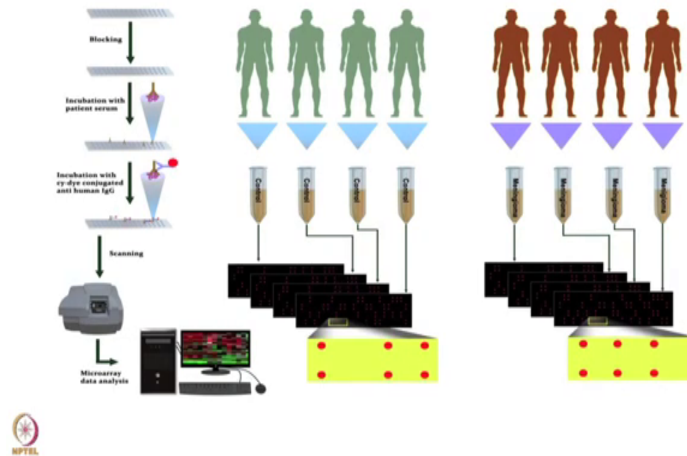
Autoantibody Biomarker Profiling in Meningiomas
using Human Proteome Arrays



So, that is the idea for this experiment where if you see on the screen we use the same workflow as I talked to you in the last lecture, you know like a western blot scheme, we have the protein arrays, we probe the patient serum sample on the chip which is having the primary antibody. And now if the antibody antigen reaction happens, then we are going to add the secondary antibody which is anti human iggs linked with the psi 3 or psi 4 fluorophores, and then you can detect those signals. In this case for the meningioma, we had the patients from the control and the grade I and grade IIs.

Another idea was can we find out some distinct pattern of the proteins which is unique for the meningioma auto antibodies. So, very briefly when we get these samples from Tata Memorial Hospital where our collaborators are there from the clinical side, before the sample come to us all the you know the patients information is available, where each patients

immnohistochemistry data is available. And based on those information they do the WHO grading.

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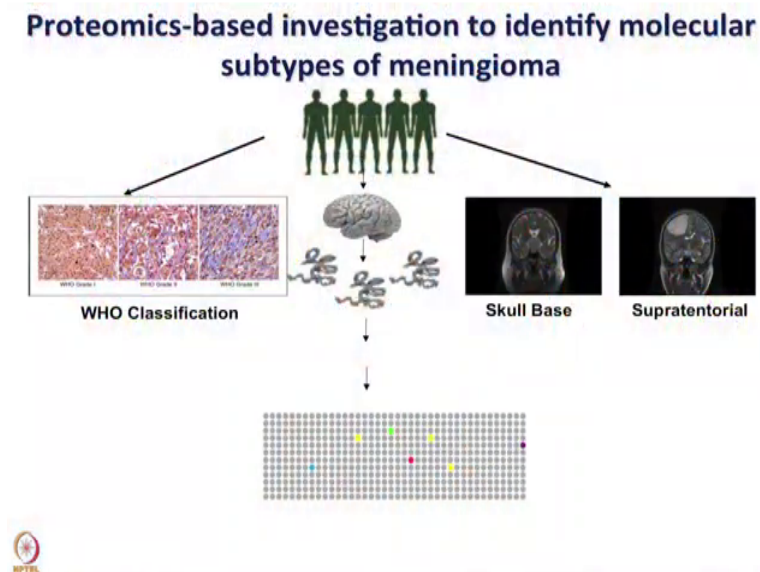

So, the patients belong to the grade I, grade II, grade III. And also their MRI images are there. So, one is looking at the radiological based features of the of the tumor as well. So, now in this case, we can see that the tumour is in which location is that built on a skull base or supratentorial.

So, while now we are going to screen the patient serum sample from the meningioma different grades, but we already have information whether the patients belong to the which grade of the tumor, and what are the radiological location of those tumors. So, once we do the screening, but as you can understand in the protein arrays we are taking one chip and one patient serum sample.

So, we are generating the data which is all distinct. So, one could reanalyze the data in different manner based on the WHO grading screen or based on the radiological based classification.

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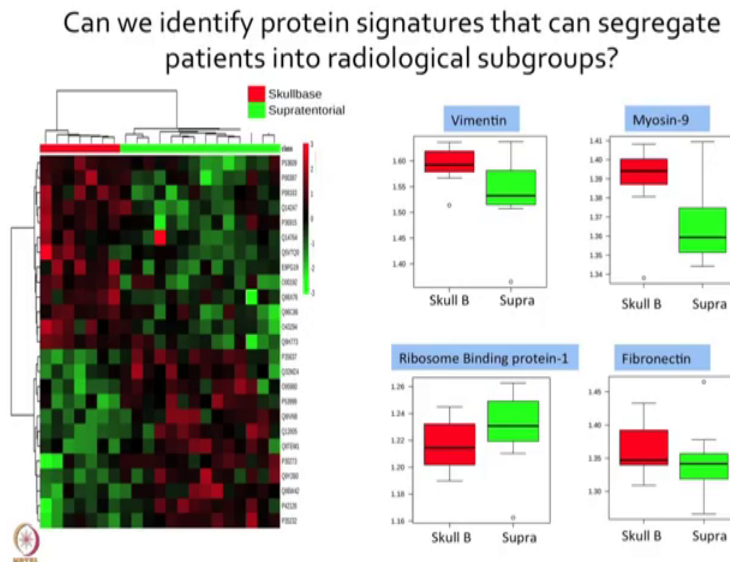

So, after doing the screening we first wanted to ask the question based on the radiological location of the tumor, can we identify the protein signature which could segregate patients into the radiological subgroups? And interestingly some of the proteins like vimentin, myosin-9, fibronectin and ribosome binding protein, they showed the different differences even based on the location of the tumor which is a skull base or supratentorial.

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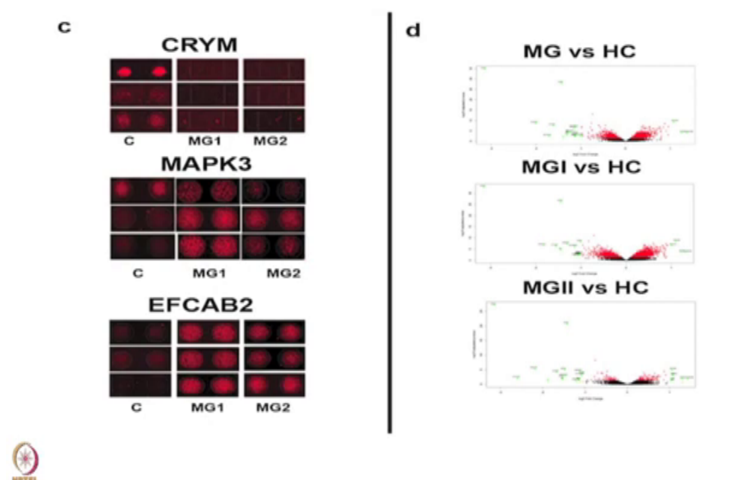

When we started looking at different grades of the tumor now build on the WHO criteria, we found some very interesting significantly dysregulated autoantibodies in meningioma patient. And you know I have shown on the in the left side image, another protein CRYM, which is highly expressing in the healthy individual, but in the disease of the brain tumor patients of meningioma you can see the no signal is seen. So, there is significant down regulation.

Whereas another protein like MAPK3 or EFCAB2 proteins, they are showing that in most of the meningioma patients, the signal is very high, very intense. So, of course, about these are the interesting observation which one would like to follow up validate and look for or they are you know the distant signal from the meningioma patients which one could you start utilizing it for the or the direction of the these kind of diseases.

On the right side, what you see volcano plots where we are after doing all the analysis, we are looking at which are the proteins emerging from the protein microarray platform which are quite significantly distinct when we compared from the healthy control with the meningioma grids.

Further, what we are looking at you know how some of these protein which are significantly disaggregated or significantly altered from the healthy individual to the grade patients can we also validate these proteins using western blot.

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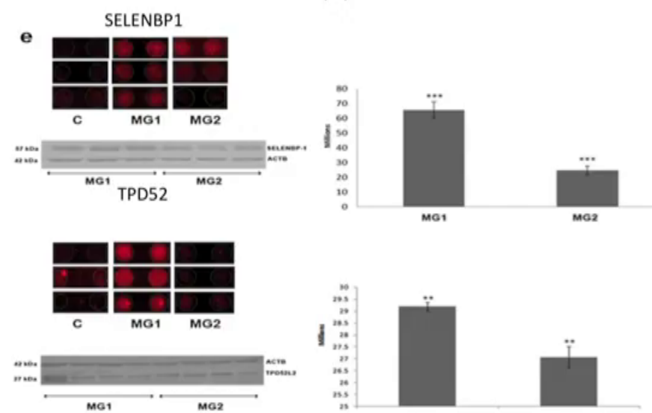
Significantly dysregulated autoantibodies in meningiomas (2)



So, for some protein which we have antibodies we try to validate those and especially if you see the selenium binding protein 1. And TPD52 proteins for those we also try to validate their

levels using western blots, and we found quite similar trends in both array platform as well as the western blot images.

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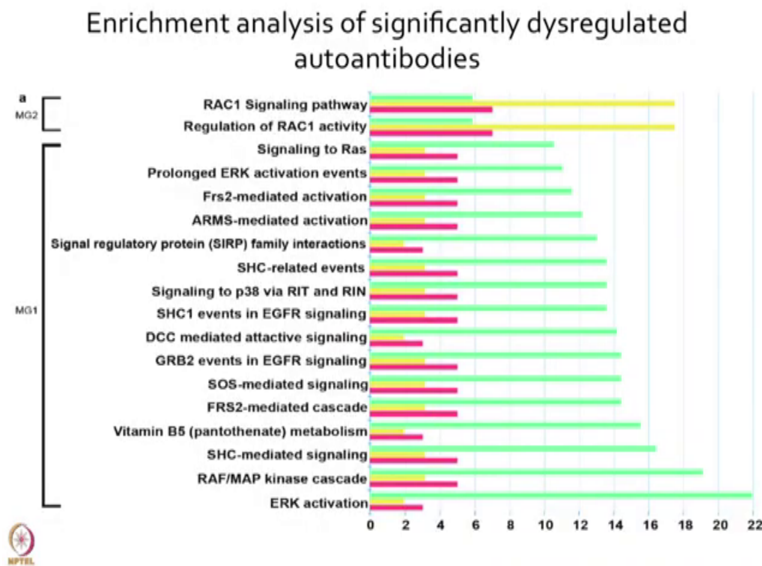

Further looking at all the autoantibodies which are changing from the healthy versus meningiomas and healthy versus meningioma grade I, or healthy versus meningioma grade II, we try to map all those proteins into different type of enrichment analysis. And we looked at which are the major pathway which are perturbed. So, we found there are 48 pathways where most of these proteins are mapping which are dysregulated in meningioma grade I as compared to the healthy controls.

And some of these pathways were quite relevant which are shown in this slide here which are based on the MAP kinase cascade, EGFR signaling, osteopontin mediated events, signaling by NGF, or signaling to RAS. Many proteins like NRAS, MAPK3, a MAPK1, PTPN11, some of

these are already implicated in majority of these pathways, and also some of them are known from other independent studies that they are quite relevant proteins in the disease biology of meningiomas.

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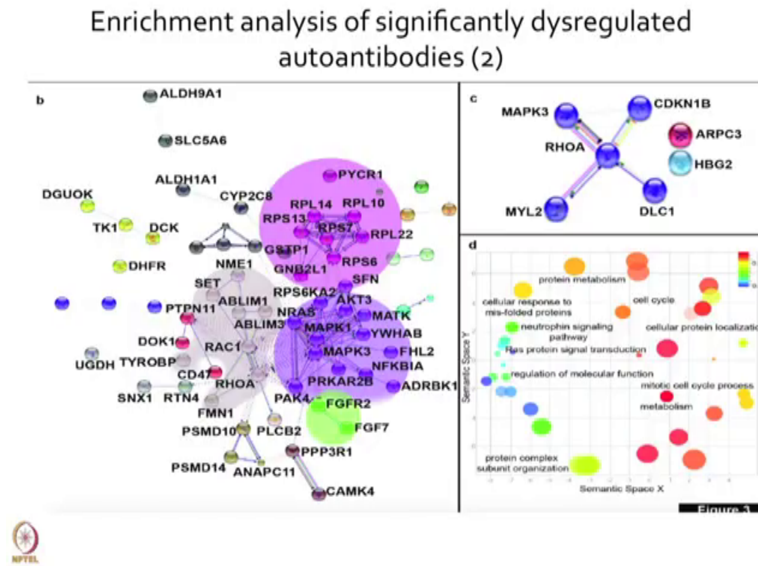

Further we try to enrich these various candidates look at their interaction networks and found that meningioma grade I gave us four distinct clusters of protein interactions. Whereas, the grade II only gave us one cluster for the looking at the protein interaction network analysis. These are shown on the left side and the top right side. Further we have also utilized the gio terms emerging from the healthy control versus meningioma grade I. And try to map these interaction networks in this figure which you see in the bottom panel.

I think this is important to appreciate the different technologies are giving us you know certain clues which are quite relevant to look for any unknown factors for that disease, but it is good

idea to also see how robust those signals are, can we measure those proteins from different platforms and still see the same pattern. So, we took the same patient samples from the serum of meningioma, and then apply those on the mass spectrometry based platform, and looked for how many of those autoantibody we can also detect when we look at the serum samples from the mass spec.

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Trends of proteins common in mass spectrometric analysis and correlation to the autoantibody response

Table 2: Trends of proteins common in mass spectrometric analysis and correlation to the autoantibody response

Proteins upregulated in MS with elevated autoantibody response

Sr. No.	Gene symbol	MG1		MG2	
		Fold change in tissue proteomics	Fold change in autoantibody response	Fold change in tissue proteomics	Fold change in autoantibody response
1	GSTP1	1.62	0.51	2.32	NS
2	C11orf67	1.77	0.52	2.38	NS
3	RPS13	1.81	0.51	1.46	NS
4	SELENBP1	1.55	0.51	2.14	NS
5	FABP5	1.79	0.55	3.83	NS
6	TPD52L2	1.35	0.60	2.54	NS
7	PDXK	0.83	0.53	0.87	NS

And this table shows that there are many protein where we could now start comparing our signal from the tissue proteomics versus autoantibody response across different grades of you know the grade I and grade II. And interestingly many proteins which showed higher up regulation even from the tissue proteomics using mass spectrometer, they were also seen higher from the serum proteomics of the autoantibody response using protein arrays. So, these are protein which are differently quite interesting and needs further investigation and validation on the larger patient cohorts.

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Trends of proteins common in mass spectrometric analysis and correlation to the autoantibody response

Proteins downregulated in MS with downregulated autoantibody response					
Sr. No.	Gene symbol	MG1		MG2	
		Fold change in tissue proteomics	Fold change in autoantibody response	Fold change in tissue proteomics	Fold change in autoantibody response
9	CRYM*	0.10	-1.44	0.13	-1.43
10	APOE	0.53	-0.54	0.61	NS
11	COX4I1	0.17	-0.51	0.25	-0.62
12	MARCKSL1	0.30	-0.64	0.42	NS
13	EPB41L3	0.48	-0.53	0.47	NS
14	RTN4	0.60	-0.62	0.65	NS
15	QDPR	0.21	-0.52	0.23	NS
16	HSPA2	0.28	-0.70	0.32	NS

Likewise, this slide show we also had some protein which you down regulated, and several of them also showed common patterned when we measured them from the mass spectrometers or from the protein array platforms.

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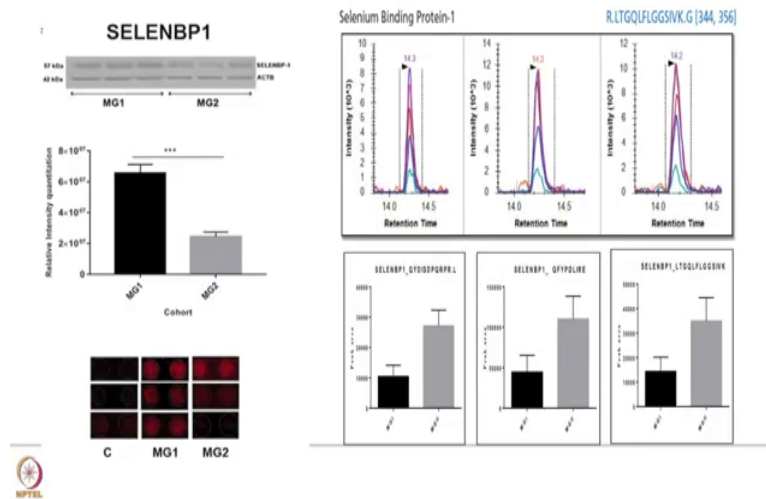

Finally idea is can we also validate these proteins. And when it comes validation, then many times we are limited with the antibody approaches. So, in our lab and you know there are many scientists working worldwide in the proteomics field, they are trying to employ different type of platforms for doing validation, and selected reaction monitoring which is mass spectrometry based triple quadrupole mass spec based assay it is coming very powerful way of doing validation.

Especially in the context of when we when we do not have antibody, then we can do this kind of measurement of several peptides and try to monitor their progression in the mass spec and look at their intensity and then try to utilize that information to look for the protein quantification.

Of course you know this whole slide and content is very complex, but the idea is to just simply give you the feel that the same protein we are trying to measure using western blot using the protein arrays and using the you know select radiation monitoring based, directed assist, and can all of them give us the confidence that these proteins are always showing the same trend when we are trying to measure them for the healthy versus diseased patients.

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Conclusions: Part-1

- Evaluation of meningioma patient sera using using high-density human proteome arrays revealed the dysregulation of 489 and 104 proteins in grades I and II of meningioma.

Proteomic investigation of meningiomas identified few candidate markers

- Filamin, Desmoplakin, Gelsolin, Annexin AI

- Autoantibody targets like IGHG4, TPD52L2 & SELNBP1 probable early detection markers

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So, from this part of meningioma patient serum sample screening for autoantibody using human proteome arrays, I hope you are convinced that it is a very robust platform which in a single short analysis can give you idea for how many proteins are dysregulated. And in this particular case, we found 489 proteins were dysregulated in the grade I, and 104 proteins in the grade II patients of meningiomas.

Then further looking at you know the much deeper biology of them different type of pathway analysis when go to start narrowing down the candidates, and we found several proteins are very interesting biomarker candidates for meningiomas like IJG4, TPD52, selenium binding protein, and we try to also do their validation using different type of proteomic technologies.

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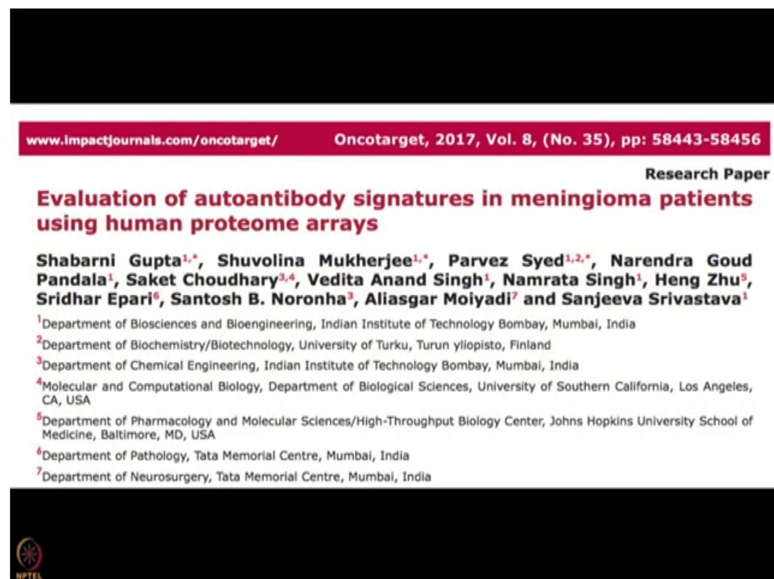
www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 35), pp: 58443-58456

Research Paper

Evaluation of autoantibody signatures in meningioma patients using human proteome arrays

Shabarni Gupta[1,*], Shuvolina Mukherjee[1,*], Parvez Syed[1,2,*], Narendra Goud Pandala[1], Saket Choudhary[3,4], Vedita Anand Singh[1], Namrata Singh[1], Heng Zhu[5], Sridhar Epari[6], Santosh B. Noronha[3], Aliasgar Moiyadi[7] and Sanjeeva Srivastava[1]

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Now, this work was published in oncotargets. Again the work involved variety of collaborators from Tata Memorial Hospital, the clinicians, some technologies from the Johns Hopkins University. And we have even within IIT, Bombay the Ph.D. students, post doctorates, and other faculty members who are bringing expertise from the you know the big data analysis disciplines. So, the entire team is then able to try to understand the complex problems. And in the last lecture, I talked to you about the gliomas, and this was the study based on the meningioma serum sample screening.

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Autoantibody Profiling of Gliomas and Meningioma CSF Samples to Identify Biomarkers Using Human Proteome Arrays

But when we talk about autoantibody screening it is always not limited only with this serum sample. So, I am now going to talk to you about of the same patients, we also had for not as many as I talked for the glioma and meningioma for the serum sample, but we also had the CSF sample from the less number of patients. And we thought can we start using the same platform of human proteome arrays, and add this cyber spinal fluid samples on the chip, and see what kind of autoantibodies we can see when we have the CSF samples.

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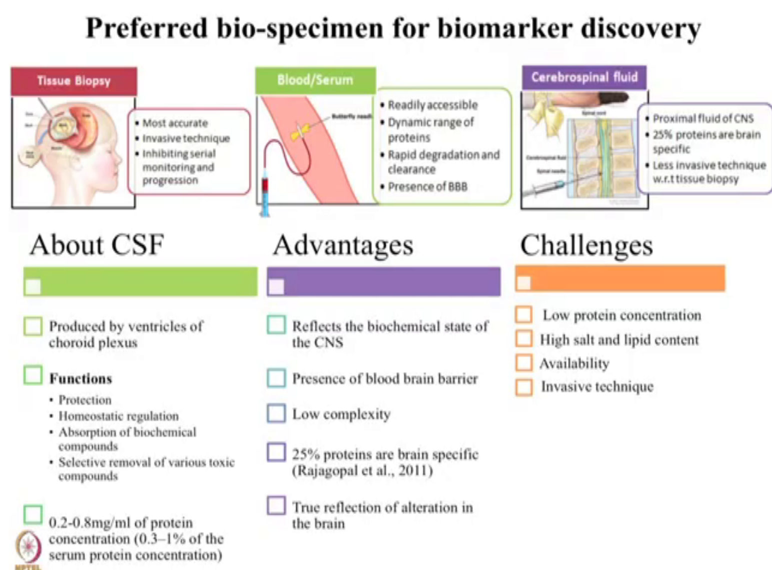

So, just to give the brief idea when we are talking about you know the screening using variety of samples, one could look for variety of you know sample type, it could be the tissue biopsies, it could be the blood serum a plasma, or it could also be cerebrospinal fluid. So, the top panel shows you the variety of options available for looking at the biomarker candidates.

But cerebrospinal fluid that is the most proximal fluid which is present in the sinus tumors and 25 percent proteins are you know very specific for the brain only. It is less invasive technique, although it is not commonly used for the you know any kind of test, but in any complications when they are taking the CSF sample this could be valuable sample for looking at autoantibodies.

CSF is very attractive, because it is closest from the tumor location. And it has a lot of advantages which is you know shown in the table here it very much mimics the biochemistry

of the brain. It is present with the blood brain barrier, low complexity, and large number of proteins have been reported which are very unique to the brain only in the CSF.

So, in some way it is very true reflection of the physiology which is happening inside the brain, but it is challenging because the protein concentration is very low, and the heart consist is very high. So, to get the right signal after removing this heart is one of the major challenge. Of course, availability of the CSF sample is not always, so one would only use that when is required for clinical conditions. And it is invasive technique, not-non invasive; although less invasive as compared to the tumor biopsies. So, I will summarize the results in a nutshell in a very brief manner.

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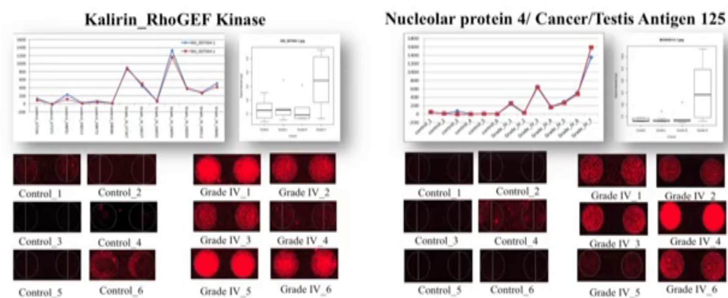

So, when we looked at the GBM patient's autoantibody response cerebrospinal fluid, we are now able to see some new proteins emerging which we had missed out from the serum

samples. And one of them interesting protein is Kalirin RhoGEF kinase. Now, this protein promotes the exchange of GDP by GTP regulates neuronal shape, growth and plasticity. And you know it is actually involved in the actin cytoskeleton remodelling. It is also detected as a driver one rotation in the other type of fashion as we see the cancer.

Other protein which looks interesting is nuclear protein 4, which is expressed predominantly in the fetal brain and testis, and localizes in the nucleolus and has a RNA binding domain. So, both of these proteins showed in majority of the grade-IV patients very high and robust signal from the CSF sample, whereas in the control they could not see much of the signal from these proteins.

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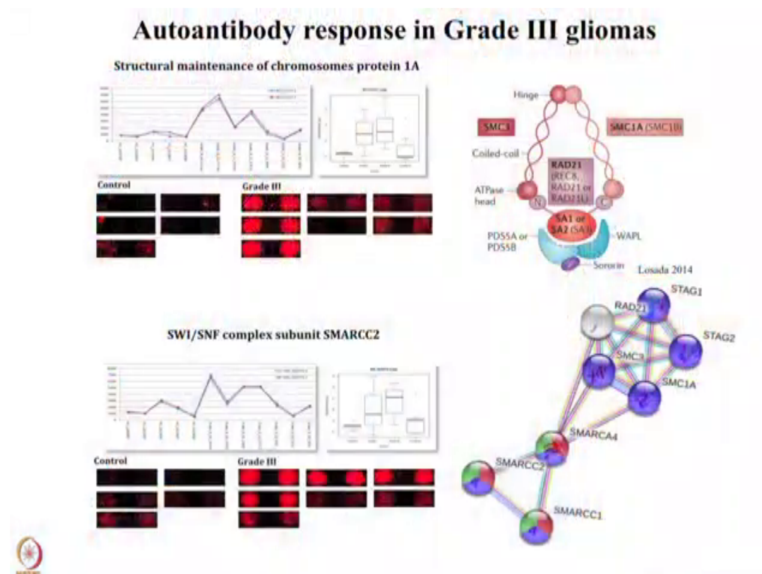

Another protein which is interesting is structural maintenance of chromosomal protein 1 A and this is the part of a cohesive complex and very important part of the kinetochore. It interacts

with BRCA1, and is involved in the DNA repair. Other interesting protein was SW1 SNF complex subunit SMARCC2 protein which is a subunit of the large ATP dependent chromatin remodelling complex.

It actually regulates the transcription of the genes by uttering chromatin structures. So, again the similar kind of workflow and data analysis strategy was employed. Here you can see lot more heterogeneity in the different patient samples and the signals, and the pre normalization the patterns are very different.

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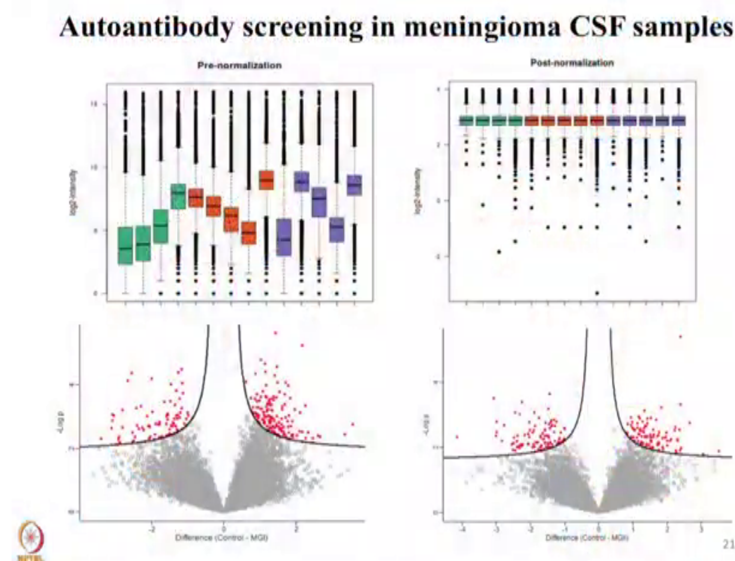

But only after post normalization when all the signal looks uniform, now we can see that which are the proteins differentially expressed with high confidence. And the below panel the volcano plot shows, if you are not normalized, you will see large number of proteins in the red which are showing the differential expression.

Whereas, on the right side, now you can see the less number of protein which are showing the differential expression after doing the post normalization. The similar strategy we applied for the CSF of the meningioma patients idea is can we now find out some new proteins which are missed out using the serum sample. And of course, we did see some new proteins emerging again.

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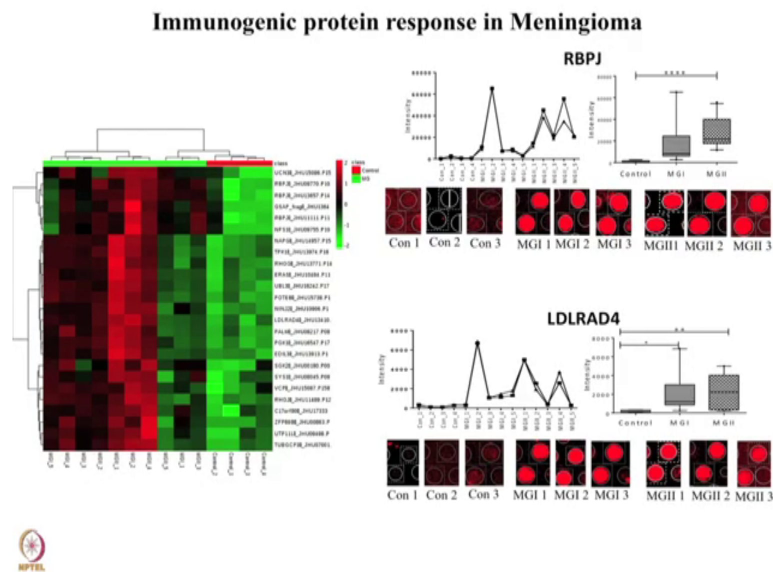

In the left side, you will see heat map a comparison of the control versus you know the meningioma patients which were from different grades. And some interesting protein like RBPJ protein which is a DNA binding protein and also principal effector of the notch signaling pathway showed up regulation in the grade-I and grade-II patients which was totally absent in the control.

Another protein which is LDLRAD4, protein which is involved in the attenuation of canonical TGF beta signaling, and shall increase abundance which is also associated with the increased cell proliferation and migration in the hepatic cancer cells not known for the brain tumors, but shown in the liver cancer. Again showed you know very clear signal, distinct signal in the grade patience of meningioma grade I, II, and III, and no signal was seen for the control population.

Then idea is can we start mapping these proteins in the interaction networks, and look at where they are showing the maximal role. And it gives at least some idea that you know many of the proteins which we are identifying they are involved in the cytokine, cytokine receptor interactions, transmembrane receptor protein also belonging to the serine threonine kinase signaling pathways.

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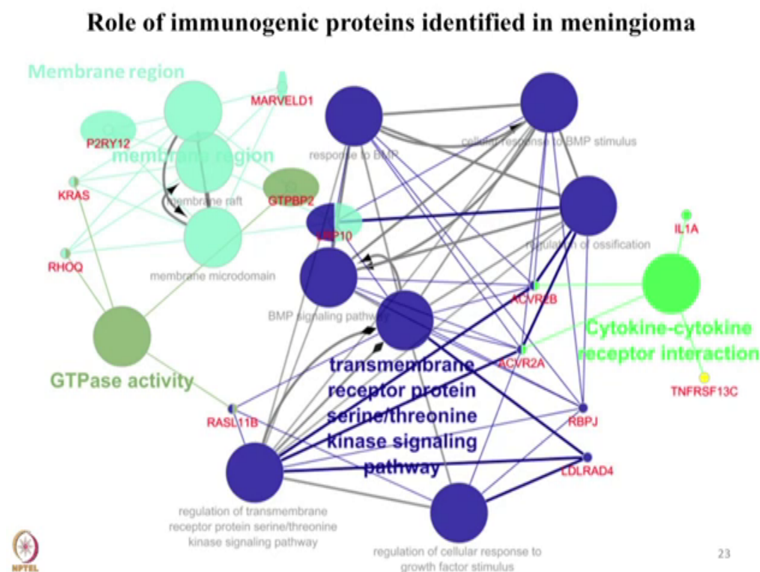

Most of them are showing some GTPase activities, also involved in the membrane regions. So, you start getting or some idea for the biological consequence of you know these proteins where they are showing their immunogenic properties. So, from this part we can conclude that few proteins show distinct response when we looked at the cerebrospinal fluid samples, both in the gliomas and meningioma. And of course, we have the panel of the protein which are quite distinct in the glioma patients as compared to the meningioma patients.

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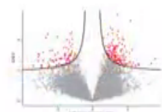
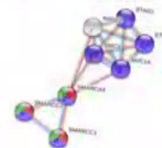
Conclusions: Part-2

- Proteins like SMC1A and SMARCC2 were found to be associated with chromatin remodelling and showed immunogenic response in grade III gliomas



- Autoantibodies against NOL4, a cancer testis antigen and Kalirin were detected in the CSF of GBM patients

- RBPJ, LDLRAD4, EDIL3, NINJ2 etc. showed significant immunogenic response in both MGI and MGII samples



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Proteins like SMC1A and SMARCC2, they are found to be associated with chromatin remodeling and showed immunogenic response in the higher grade patience of the gliomas. Autoantibody response for the NOL4 protein, which is a cancer testis antigen protein and kalirin protein was detected in the GBM patients. Few proteins like RBPJ, LDLRAD4,

EDIL3, NINJ2, they showed significant immunogenic response in both meningioma grade-I and grade-II patients.

I hope now you are convinced that protein arrays could be a very powerful way of looking at autoantibody responses from the serum and cerebrospinal fluid. Anyone could look at different type of autoimmune diseases; one could also look at even different type of cancer and complex diseases where there is possibility of autoantibody production.

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Protein-protein interactions using NAPPA arrays

Now, let me shift gear and very briefly talk to you about protein-protein interactions using NAPPA arrays. So, most of the study which I talked to you was essentially on the purified proteins printed on the chip, and seventeen twenty thousand proteins were printed and those represents the maximum proteome coverage for humans, so human proteome arrays.

At the same time the same kind of arrays could also be utilized for studying protein-protein interaction, but protein-protein interaction becomes more you know interesting if you think about how best you are going to look at the protein expression in its you know most native condition possible, and that is what I think NAPPA arrays are provide a much more powerful way.

Because we are expressing the protein from the DNA directly on the chip in situ and then think about an experiment where when you are you know the query protein for which you want to measure the interaction, if that proteins also is the DNA and going to get expressed along with a protein when you add the in vitro-transcription translation mix, then that will be very powerful.

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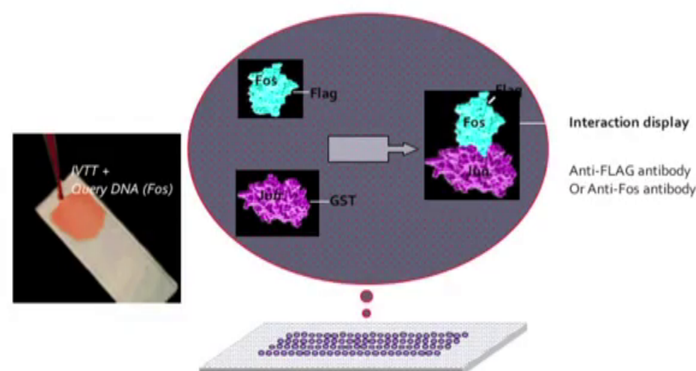
Protein-protein Interactions:
Co-expression (Jun-Fos interactions)



Ramachandran, N., Hainsworth, E., Bhullar, B., Eisenstein, S. et al., Self-assembling protein mircoarrays. Science 2004, 305, 86–90.

So, I am just showing you this image which talks to you about studying protein-protein interaction using NAPPA arrays using co-expression analysis. Here as I mentioned idea is that on the chip you have let us say 10,000 spots which are all having the CDNA, and you are going to express the protein using in vitro transcription translation mix which we talked earlier the cell free expression based arrays.

Now, when you are adding your query protein rather than adding the purified protein, you are simply adding the DNA of that query protein. So, in this case, we are adding the DNA query for the FOS protein. But keep in mind that now you have to have some strategy for the measurement of the interactions. So, now idea is if the FOS protein is you know with the DNA is going to come and bind on the chip, it will going to show interaction with the JUN protein which is not interacted and might be showing interaction with some of their protein as well.

So, how to measure these protein? So, the FOS in this case was having the flag tag, but the all other proteins on the chip is having the GST tag. And based on this if all the proteins are expressing the proteins on the chip and the DNA from the FOS, then now we are going to have the proteins expressed on the chip, and going to show the interactions where it is going to show the binding. This is an idea for doing the co-expression based experiment.

So, a similar experiment we were also teaching some students at the Cold Spring Harbor Laboratory in one of the previous courses which have conducted Dr. Josh Liber. You have heard Josh talking about different type of applications. I am trying to convey you here that these experiments are not very difficult to perform, and these experiments were done with the participants in a workshop which we conducted without having the you know kind of you know big laboratory setup over there. We took these arrays which having the CDNA printed with the NAPPA chemistry, and the expression was performed in the course by the participants themselves.

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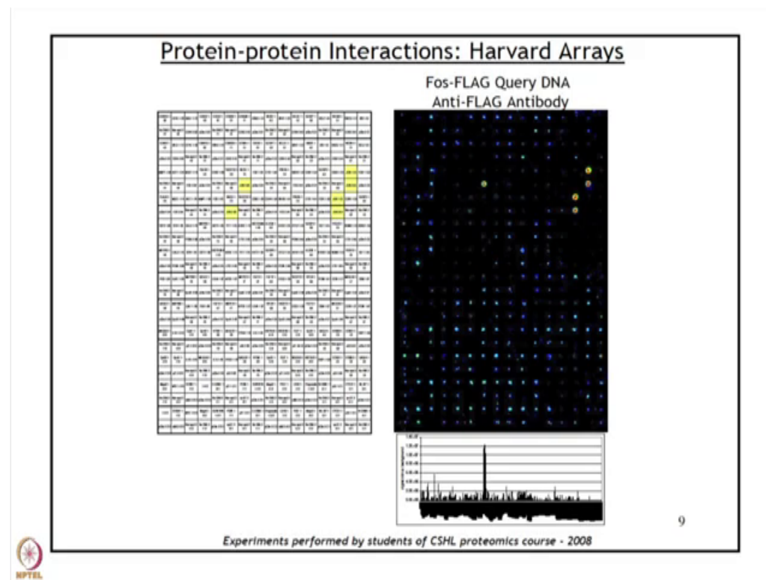

And idea here was the same experiment can we use you can see the layout of the chip on the left side, where we are hoping that you know the FOS is going to bind to the Jun with the yellow spots which are seen. And on the right side, you see after doing the actual experiment, we could see those signals for the duplicated spots of the Jun showing the binding with the FOS query protein.

We also looked at measurement of anti flag antibody, and can be now measure the where the FOS proteins are binding, and based on that we could now measure the signal and look for the quantification of that.

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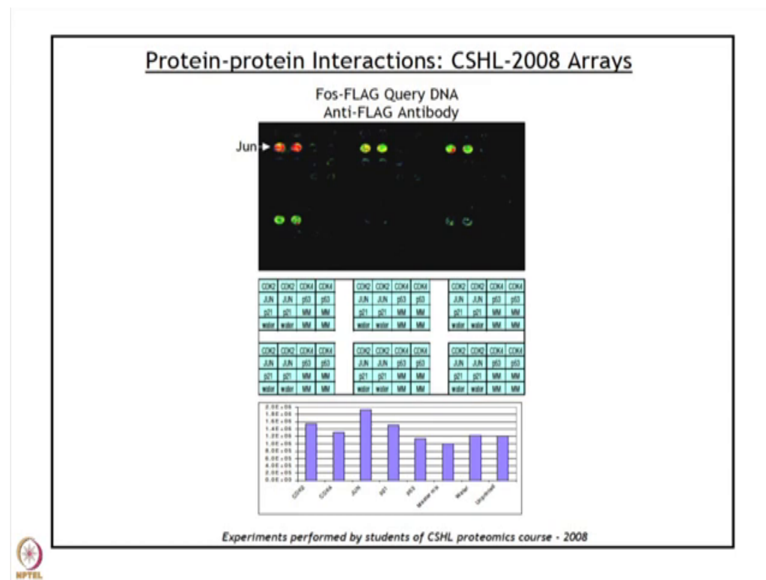

So, this shows another small array which again you know idea is to just show you the crude data, the small raw data, which even can be obtained in the small setting in a workshop which participant themselves are able to do these experiments. And in this case again with the FOS flag query DNA which is after co-expression going to bind to the Jun protein, one could start measuring the signal. And you can see these are the duplicate spots which are seen for the Jun protein.

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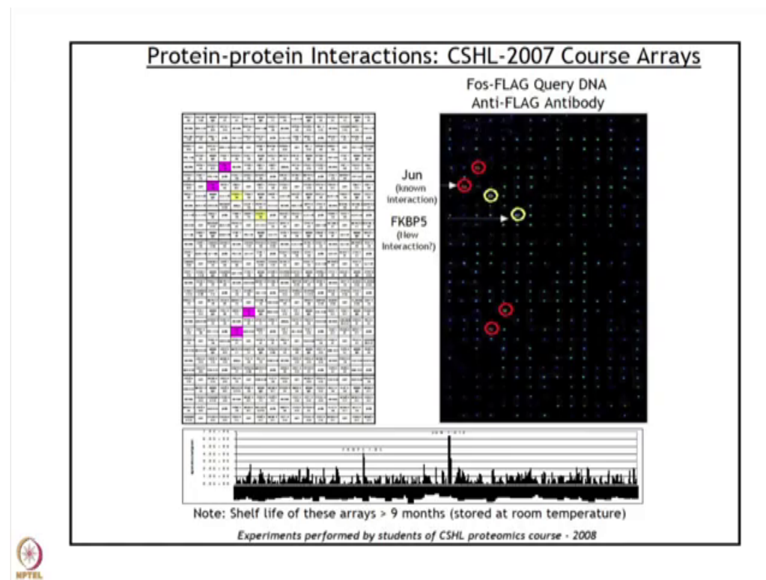

And likewise, we had another experiment here we showed that you know now when we are measuring the binding with the FOS, not only that you know the FOS protein which is you can see in the red is binding with the Jun, but also we could identify some new interactors. And in this case FKBP5 was shown as a potential new interactor where the FOS protein was binding. Additionally now these arrays could be done for use for the other applications as well especially to look for the kinase assays.

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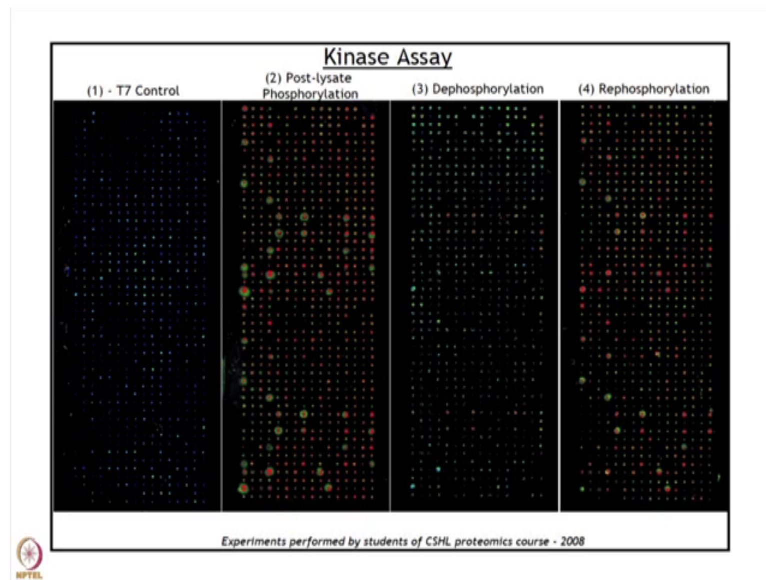

And Josh as talked to you about various type of phosphorylation experiments, but I am just again showing you this experiment done in the course for by the participants, where they took these NAPPA arrays did the phosphorylation experiment dephosphorylation and rephosphorylation.

Idea was can we monitor some of these kinase proteins and look at their expression, and because we do not want to have any confounding effect coming from their lysate (Refer Time: 25:07) or IVT mix that is why we are doing dephosphorylation strip in between, and then again rephosphorylation to ensure that those phosphorylation what we are measuring though they are actually coming from the actual activity of these kinase proteins. So, after doing that, then we were able to measure the signal for you know the controls and dephosphorylation, post lysate phosphorylation, rephosphorylation of these proteins.

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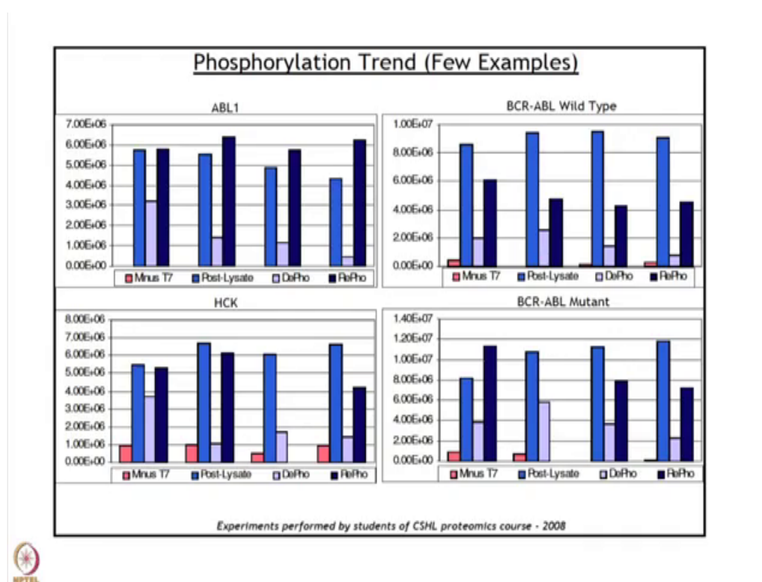

And some proteins you know look quite interesting when we start measuring their trends for the ABL protein, BCR-ABL mutant we had on the chip. And you know just it gives you the idea that lot of interesting biology could be studied very rapidly if you know how to utilize these kind of array platform to address biological questions.

So, I hope I was able to convey you that you know protein arrays could be utilized for different type of interesting biological applications. Looking at the clinical context to that one could look at autoantibodies screening, looking at the you know protein of interest one could do protein-protein interactions, one could also look at you know various type of activity for the PT modifications like kinase activity, and different ways of arrays can be utilized from the human proteome arrays to the NAPPA arrays.

So, protein microarrays are definitely a very powerful platform for various applications specifically for measuring the protein-protein, and protein other biomolecular interactions, post translational modifications and biomarker discovery.

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Protein Microarrays: Summary

- Protein microarrays have been used for various applications (protein-biomolecular interactions, PTMs, biomarker screening), which is increasing over the years.
- Autoantibody screening for biomarkers using serum and CSF samples could be performed at proteome-wide scale using Human Proteome Arrays, which is otherwise not possible from MS or other techniques

Autoantibody screening definitely show the power of using protein arrays directly from the crude serum or CSF samples, we are able to screen this kind of you know the autoantibody response which is very close to the clinical type of testing which is not possible using very robust mass spectrometry based platform. Mass spec are you know advanced instruments, but for doing the analysis you need to extract the protein out from serum and CSF samples, clean them up, digest them, get the peptides out, and then only analyze the peptide, then do lot of analysis.

Now, while you are you want to analyze the serum or CSF, you have to also do additional step of depletion, you want to remove the abundant proteins, and then only you will be able to get the right signal. So, therefore, using arrays and directly by using the crude serum or CSF sample shows you the you know how powerful this platform is, you will you are able to measure the signal which is not possible from other technology platforms,

I hope you know you are learning these techniques for in the field of interactor mix and bring it a sciences, but also able to appreciate where each of the technology gives you more advantage and where the technologies have limitations. I hope you will start studying more about not a specifically only protein arrays, but variety of proteomic technologies, and start thinking about which technology can address your better questions of interest much more powerful and robust manner.

Thank you.

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Points to Ponder

- Meningioma are primary brain tumors arising from the meninges, the layer covering the brain. These tumors are characteristically very different from gliomas
- The procedure of identifying tumor associated autoantibodies in the biofluids is very similar to that of western blotting
- Proteins showing immunogenic response can further be validated using different techniques like western blotting and mass spectrometry based platforms to look at the differential abundance of the protein in different cohorts
- The significant proteins showing antigenic response can be mapped into pathways to understand their biological role and that in turn also may also help in understanding alterations that occur at the very early stages of neoplasm formation