**An Introduction to Proteogenomics**

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**Lecture – 13**
**Applications of Proteomics**

Welcome to MOOC course on Introduction to Proteogenomics. In the last module, we first studied about genomic revolution, genomic technologies. And second module we have started discussing about proteomics. In my last two lectures, I have tried to give you a very broad overview of proteomics field, different technologies which are being used in my first lecture, and a second lecture try to focus more on mass spectrometry based proteomics.

Today I am going to talk to you about some of our ongoing research work to give you the flavor of how proteomics technologies could be used for different type of applications, especially more focus on clinical proteomics that in some way is going to summarize that different type of proteomic technologies could be used to address different biological questions. As we go along after this lecture, there will be much more focused discussions about mass spectrometry based proteomics, different software's and tools involved by various experts of the field we are going to talk about much for detail about how to use various tool for data analysis. So, this is the third lecture of giving you the basics and overview. After this there will be much more focused discussions and the hands on directives of the workshop.

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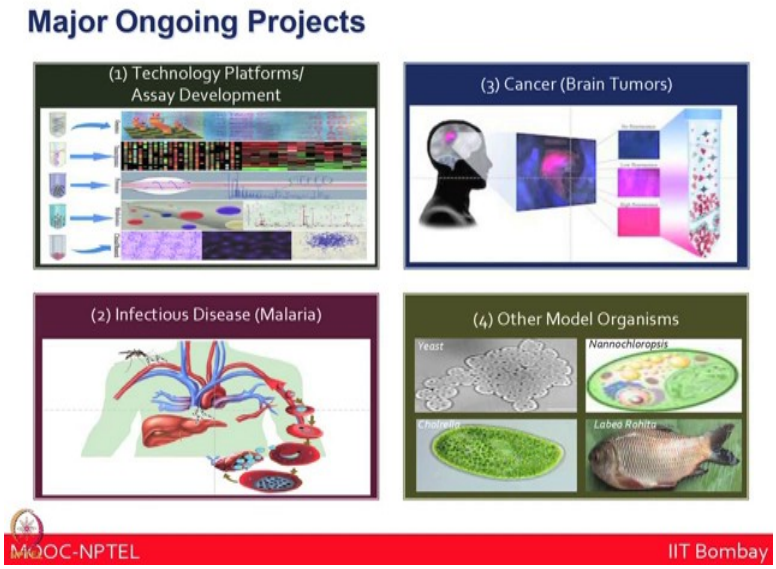


So, just talking about various type of ongoing projects especially in my laboratory at IIT, Bombay, we look at some of the technology platform or the assay development as one of the areas of interest. A major group of the lab works on the infectious disease, especially malaria and dengue. And try to look at different type of either a diagnostic biomarkers or looking at the prognosis how a disease transformed from the non-severe to the severe form, and how using proteomics one could try to understand that.

Additionally a major focus in the lab is on the brain tumor especially cancer biomarkers in collaboration from a Tata Memorial hospital and then we have various ongoing projects from different collaborators on other model organisms. For this you know brief presentation, I will focus mainly on two different a disease projects one on the infectious disease and other on the cancer projects. And try to give you a you know a broad understanding about how proteomics technologies could be used to study different type of you know clinical problems.

Let us first talk about infectious disease or malaria proteomics project. Malaria, I am sure all of you are familiar. You know as the monsoon season starts people start getting you know different type of mosquito bite and get affected from either you know falciparum malaria, vivax malaria or sometime dengue fever, Chikungunya variety of fever you can term, and it becomes very different for the clinicians and the doctors to really you know

make accurate detection of what kind of you know the organism has caused this particular type of fever.

And if you get the right you know diagnosis only then you are able to get the right type of treatment, right. So, the interestingly looking at malaria, the trend for malaria pathogens have slightly changed over the time period, especially last you know couple of years when we have started looking say it this problem in India, we found that falciparum malaria have reduced, whereas vivax malaria has you know quite increased now.

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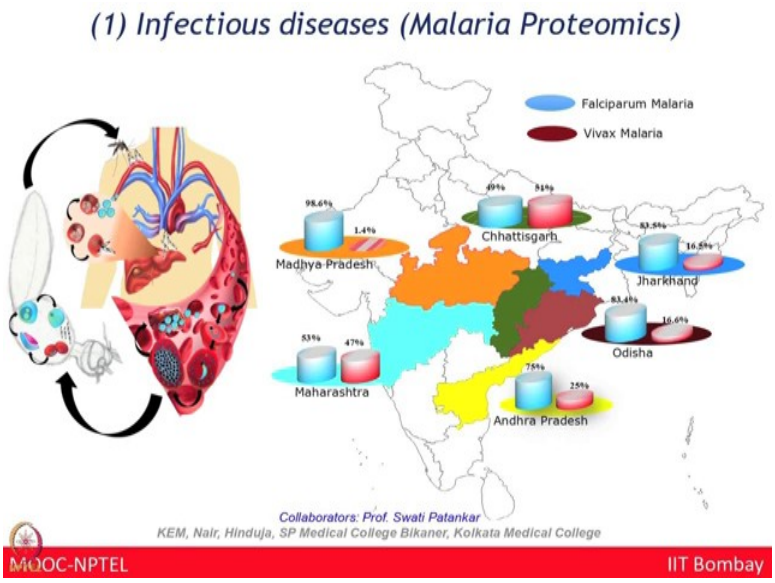


And you can see the trends in different regions of India, where it shows that now vivax malaria is pretty much on rise. While earlier this particular you know vivax malaria was thought to be you know very benign and not you know causing much of the problems for malaria. But now looks like you know it is one of the major culprit which is you know causing lot of malaria problems in whole India and of course, many parts of the world as well.

So, however, our major understanding of malaria predominantly depends on the falciparum malaria, lot of research has happened in that particular pathogen. But when it comes to vivax it has been very limited because of various reasons of you know our inability to culture the vivax in the culture system which is not the case for falciparum. And also the you know vivax here at a very low parasitemia level, it could cause even

you know the severe effects which is not the case again for the falciparum. So, current modalities of diagnosis of you know malaria parasites based on the microscopy or it could be you know rapid diagnostic test or it could be PCR.

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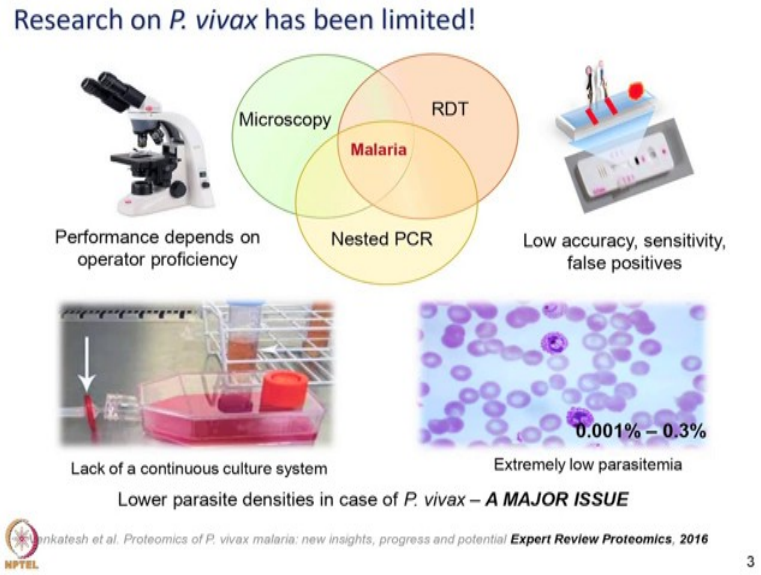


Why microscopy is gold standard when the, you know pathologist they look at the you know the parasites in the microscopes. And as he has a trained eye who can look at the parasites you know well, I think that is you know very accurate ways of detecting parasite. RDTs are very quick, these are you know based on some antigens when you can do some strip based test quickly to tell if a pathogen you know is coming from the falciparum or vivax, or mixed infection and one could you know try to get that information. Or the PCR could be utilized to be very you know accurate that you know which kind of pathogen is causing in this particular febrile response.

However, each of these methods have their own you know pros and cons. Especially RDTs have lot of you know fast positives, PCR can be done only in the specialized labs, so it needs lot of you know further testing. And overall limitation for the whole field has been that most of our information is based on falciparum not vivax. Therefore, everything is you know we try to put together based on the falciparum and say is that falciparum positive or negative, and accordingly the whether this could be vivax. So, there is still need to look at the specific antigens for vivax if we really want to investigate this problem.

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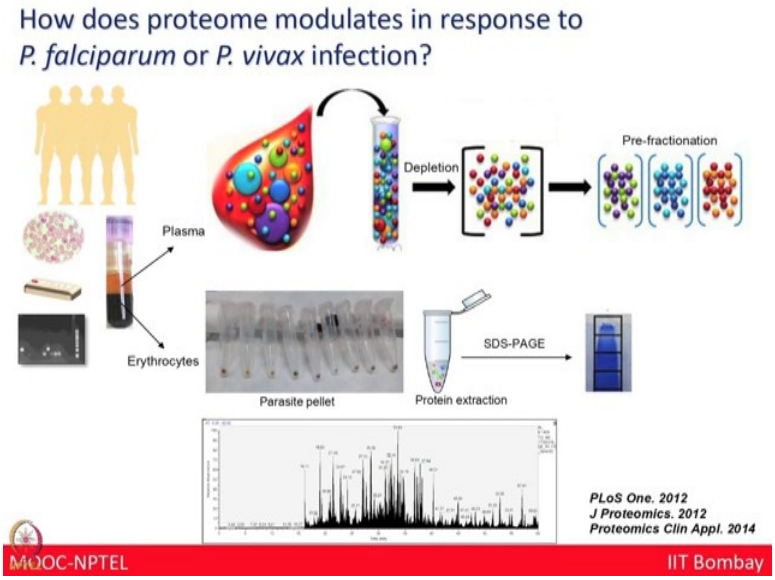


So, in this slide, one could look at different type of proteomic approaches different type of you know biospecimen to try to investigate this problem. Let us say one question we may try to address how does the proteome modulates in response to plasmodium falciparum or vivax infection. If you do that, we looked at you know the patient's blood sample, and you know try to look at the plasma as well as the erythrocytes. The plasma proteome to look at the host responses and erythrocytes for looking at the parasite protein to look at what are the parasite proteins which one could detect directly from the clinical isolates.

So, both of these is strategy then someway are complementary to give us you know very vast information about the parasite proteome and the host proteome to find out what is happening in response of this malaria in these patients. So, one of the goal of the project was to look at can we identify some you know specific biomarkers which could be very unique for the vivax malaria, a lot of work has happened in the lab.

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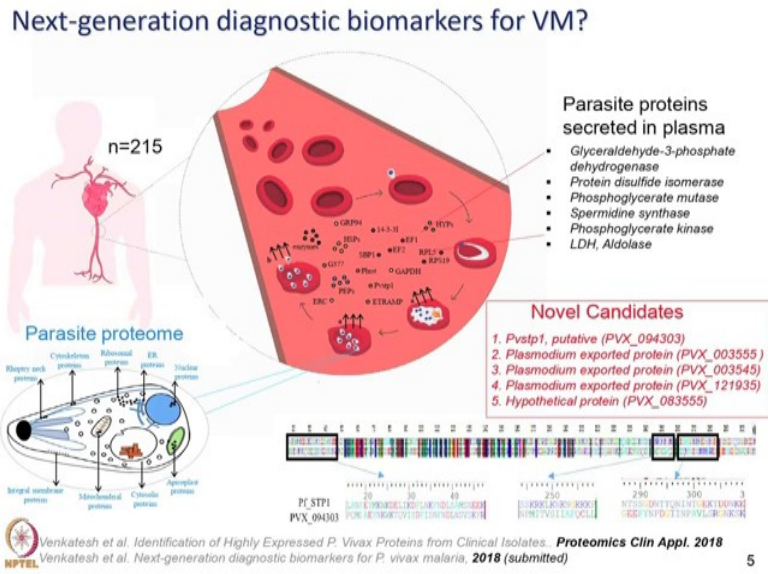


But this in a nutshell in one of the study where over 200 patients were you know enrolled. We looked at parasite proteome. We also look at which of the parasite proteins are secreted in the plasma, because those are circulating in the blood and those could be much more relevant biomarkers for you know our study. And therefore, some of these biomarkers we try to now you know do the bioinformatics analysis and see are they only uniquely present in vivax or which is also present in the falciparum.

And interestingly many of you know at least five candidates looks very promising only present in the vivax which could be next generation diagnostic biomarkers, now there is a need to take these leads forward to clone, purify these proteins, and then see with the possibilities of having the RDTs based on these protein biomarkers. Additionally we looked at now in protein microarray based platform to know which antigens are markers of exposure in malaria.

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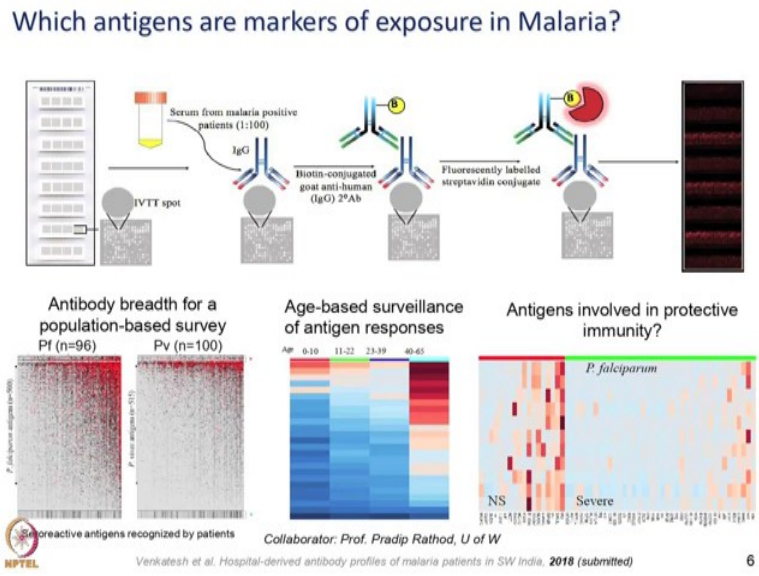


To look at this you know the some of this study can be foundation for doing the population based studies, where one could ask various questions, what is the antibody breadth for a population based survey. And in this case, we looked at from the Goa population in collaboration from the history of Washington preserved Pradeep Rathore's lab. You know variety of patients affected from malaria both falciparum and vivax, we try to screen, and on the chip we had only the antigens from falciparum or vivax parasites.

So, now the patient's serum samples are probed on the chip, and we are looking at antibody breadth of the patient suffering from falciparum or malaria. Also we try to look at the surveillance kind of responses based on the antigens which is shown in this particular you know the middle segment here, where you can see we are going to based on the age group, one could try to see different type of responses. And in the left side, you can see based on the falciparum or vivax, the patterns are different for the you know seroreactive antigen which I recognized by these patients.

And finally, if you look at the right panel, we are looking at also the severe and non-severe responses in these patients, and you can see very different pattern from the non-severe versus the severe patients. So, again this could be one of the you know interesting approach of looking at the antibody based responses using protein microarrays.

Again to address what are the mechanism of vivax causing the severely for which we do not have much information, and some of these study I am just talking to you about you know partially published and some are lead and still unpublished, where goal was to look at if a patient comes to a doctor, and there you know having the mild fever. Whether one could try to look at possibility of them transforming to the severe form, and what kind of proteins and metabolites could be indicative of the severity.

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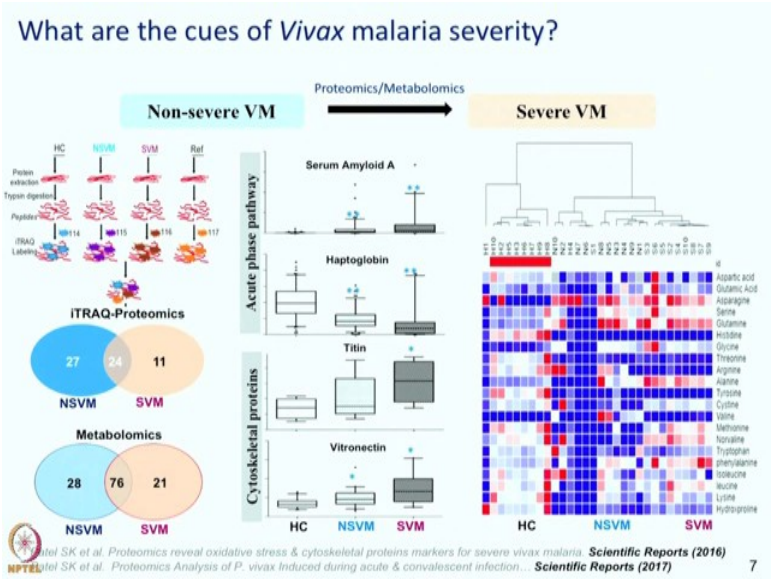


So, in this slide, we did a quantitative mass spectrometry based workflow. When the healthy individuals, non-severe vivax malaria patients, severe vivax malaria patients were screen along with them we use a reference pool here. Sort of this you know a typical iTRAQ based mass spectrometry study which we talked in the previous lectures was implied, and what it revealed that there are set of proteins which are very unique to non-severe or the severe vivax patients looking at both the proteomics approach.

And even after metabolomics also we found that there are some very unique metabolites which are secreted here. Some of the proteins from the acute phase pathway and the cytoskeletal proteins are shown on the screen here. Then a protein like Serum Amyloid A shows from the healthy individual to the non-severe to the severe patients kind of you know rise of the chain is abundance of these proteins.

Another protein like Haptoglobin shows reduction overall the you know the concentration is reduced from the non-severe to the severe patients and that is you know

more logical because you know the Haptoglobin and Hemoglobin. They form the complex and therefore, you will see the reduction of this particular protein in the severe vivax patients.

Some of the cytoskeletal proteins like Titin and Vitronectin, they showed the increased response as the disease progresses from the non-severe to the severe type which is indicative of some of the muscle protein and cytoskeletal protein being you know secreted from the muscles to the blood stream, and that shows you know the now the patients could be modulated from the non-severe to the severe type.

We try to look at various metabolites shown in the right side and then the red dot shows you the pattern of healthy individual, and the you know the this part shows the non severe, and the last part show the severe pattern. Even by looking at you know the metabolite profile one could see at least there are some metabolites, especially whereas amino acids they show the change in response to the non-severe and severe type of malaria.

So, based on this, we are trying to now capture which are all changes one could you know utilize from the omics technologies, and try to look at what is the mechanistic insight of vivax malaria severity. We have tried to put together lot of information from proteomics data and metabolomics data. Aim is not to give you lot of detail right now for the mechanism, but you know I am trying to emphasize that how these tools and technology what we are talking can give you the new insights which could probably help you to understand the mechanism of a given disease.

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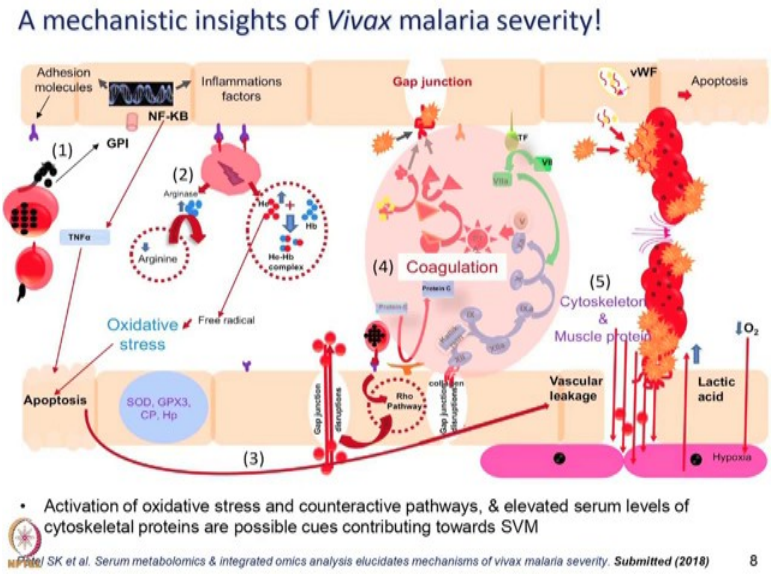


So, in this context, you know very first time we are now able to put together various pieces and trying to see that in which way the various changes which are happening which are reflecting from the additional molecules to the inflammation factors, how they are you know with the change of various type of amino acids. And change in the you know Haptoglobin level, then you know leading to the oxidative stress or apoptosis followed by it is influencing the vascular leakage, and coagulation pathway which is eventually causing variety of you know severe infection which we can see.

So, the activation of oxidative stress as well as the you know the cytoskeletal regulatory molecules could be the one of the major mechanism which is contributing towards the severe vivax malaria. Of course, this is very you know complex slide which leaves out more time, but I am not going in detail. Here idea is only to give you the, you know the glimpse of what you can try to understand from the proteomics and other omic technologies.

Additionally, we are trying to also addressed you know more focus questions like in case of falciparum malaria, there are various type of severe infections happens like you know patient could be suffering from the you know cerebral malaria or renal failure or variety of other complications. All these could be termed as SFM or severe falciparum malaria. And question was could we look at proteomic based alteration in the severe falciparum malaria based complications.

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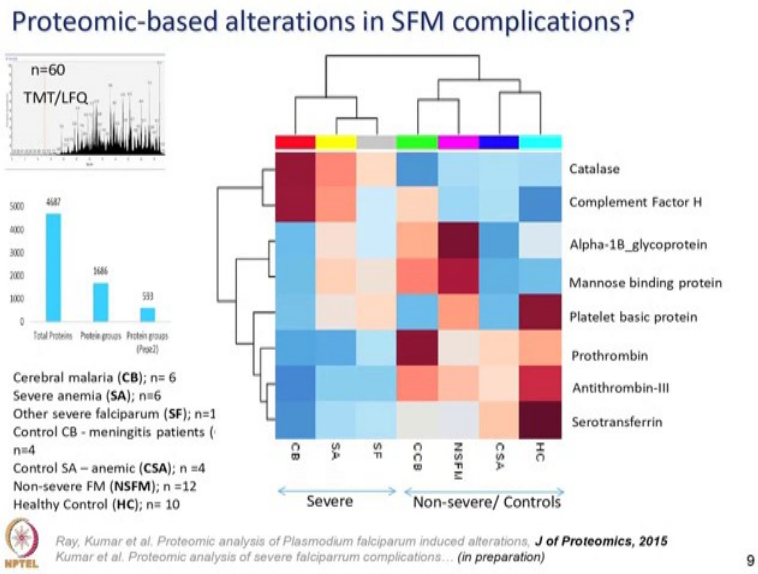


In this slide we selected the patient from cerebral malaria, severe anemia, other type of severe falciparum type. When we do these kind of proteomics studies or clinical studies, choosing the right control becomes very crucial and of course, challenging as well. So, in this case for the you know anemic population we choose as a control for severe anemia, we also choose various type of non-sever falciparum patients, we got some meningitis patient for the you know control as cerebral malaria. So, you need to have the right type of controls to compare the various types of disease complications.

Now, after doing lot of proteomics work flow, this heat map is shown here which shows that you know one could actually segregate severe and non-severe falciparum based on some of the proteins showed on the right hand side. And even within each type of severe infection like you know cerebral malaria, severe anemia, other type of you know a severe infections, their trends for virus proteins are quite different. And therefore, this information could be helpful to get a glimpse of what type of severe infections these you know patients might be undergoing if they are affected from the falciparum malaria.

Additionally, we are also interested to look at are there some parasite protein secreted in the serum or plasma of these individuals, and whether those could be used as a next generation diagnostic biomarkers. Because you know some of the existing biomarker like PFHRP2 while it has been you know good lead for the RDTs, but there are many population where now it is being shown that you know there are some mutations

happening and this may not be the best diagnostic biomarker. So, this definitely need to have an alternative next generation diagnostic biomarkers even for falciparum malaria.

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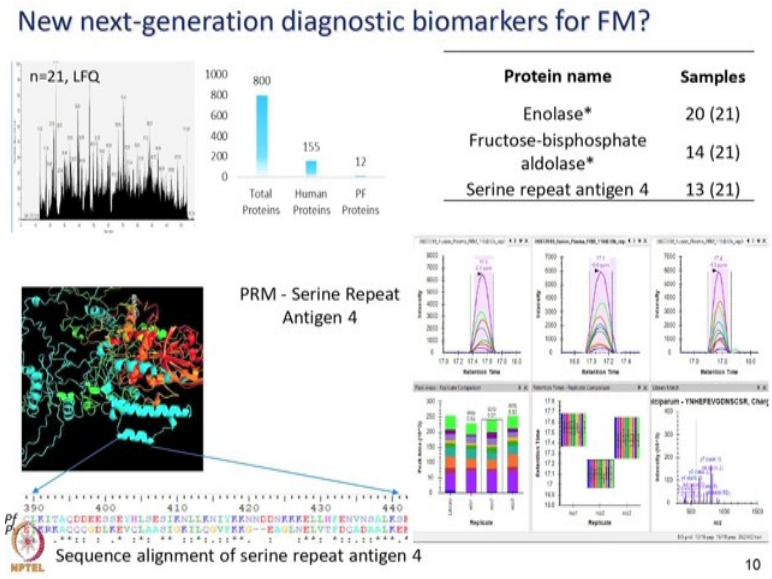


And in this slide you know various proteins which we try to identify, some of them they are already you know same protein, but are available in the existing RDTs. But in additionally we are also able to find some new proteins which are secreted from the parasite in the host that is stream or the plasma. And one of those is you know serine repeat Antigen 4-protein which looks promising again and could be taken forward as the potential biomarker candidate.

So, in the nutshell of the malaria project, we now have mapped various leads various protein targets both from the parasite as well as from the host. And our aim is to look at you know what happens to the given protein like let say Alpha 1-antichymotrypsin or alpha 2 which is glycoprotein or Complement C3 across different type of complication from the non-severe falciparum to the non severe vivax to the dengue fever and severe type of falciparum and vivax.

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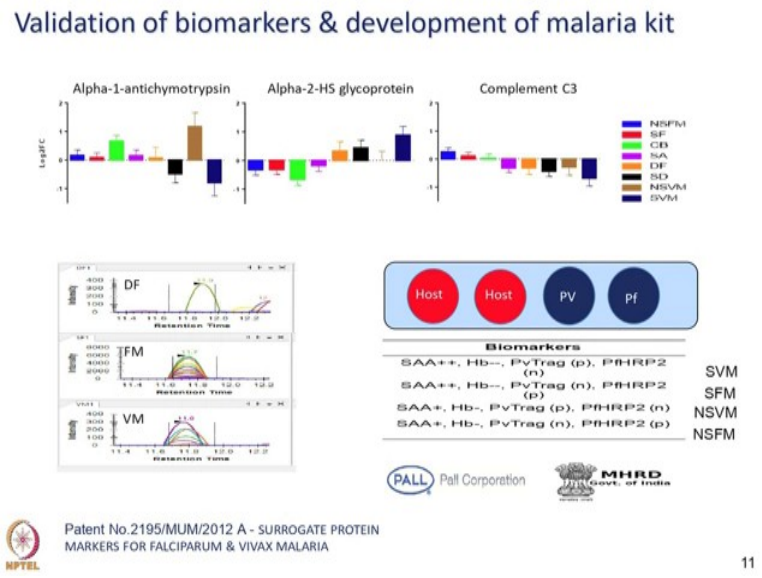


So, what happen to the same protein across multiple infection, and if there is a real you know signal coming out of a in a specific type of infection, you will see a different trend. Now, some of this information we are trying to take forward to develop some of the, you know the possible kits or assays for the better diagnosis and prognosis of the patients.

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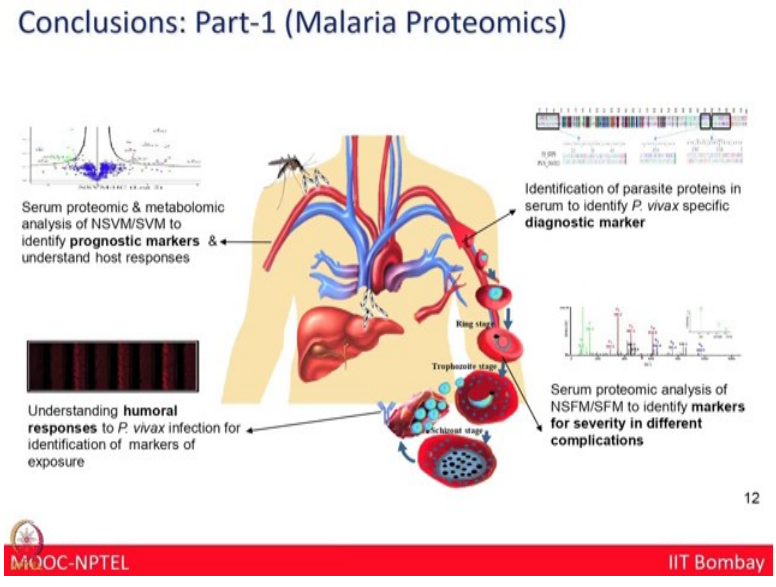


So, in general the conclusion for the first part of malaria proteomics is we are looking at you know various approaches of serum proteomics and metabolomic analysis to look for the prognostic biomarkers and understand the host responses. We are also looking at

various type of parasite proteins in the serum to look at the potential biomarkers for the diagnosis.

We have also investigated you know the non-severe to severe comparison to try to look at the severity of different complication both in the falciparum and vivax, and actually nothing was available information for the vivax. And we have also done much more in depth investigation of the different type of severe falciparum complications. And you know an offshoot of the overall project is also looking at the antibody breadth, and looking at humoral responses of you know the using the protein microarray based work flow to look at various type of you know markers of exposure of malaria.

So, this is you know where you can see to investigate one clinical problem We can realize different type of technologies from gel based to mass spectrometry, to micro arrays and to a SPR even to test out the protein the interactions to then try to understand comprehensively a given problem or a given system.

So, let us now shift gear from infectious disease moving onto one of the clinical problem of you know great relevance of the cancer, especially the brain tumors which is very deadly tumor which affects you know lot of patients, who do not survive after you know very long if you are the diagnosed with the brain tumors.

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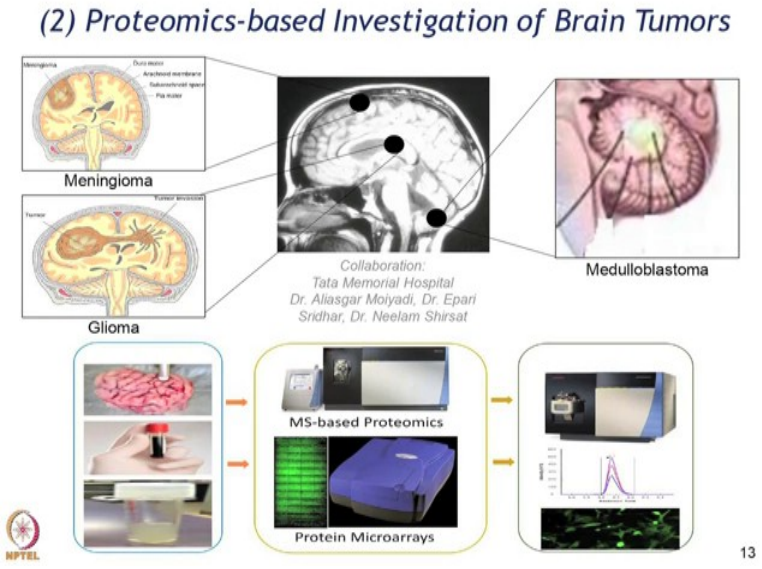

Depending on the location of the in which region the tumor is the brain tumor could be termed as a gliomas if they are derived from the glial cell or meningioma, if they are derived from the meninges or medulloblastoma, if that is a pediatric tumors. So, due to this kind of project, we collaborated from doctors from Tata Memorial Hospital as well as you know ACTREC in Mumbai. And we try to utilize various type of work flows and approaches how to use proteomics to investigate these problems.

But let's kind of you know give you brief of glioma than meningiomas which are very challenging brain tumors. Most of the you know these brain tumors are very heterogeneous as well as if you look at the available information from WHO that is more based on the cell morphology and some of the immunohistochemistry based biomarkers.

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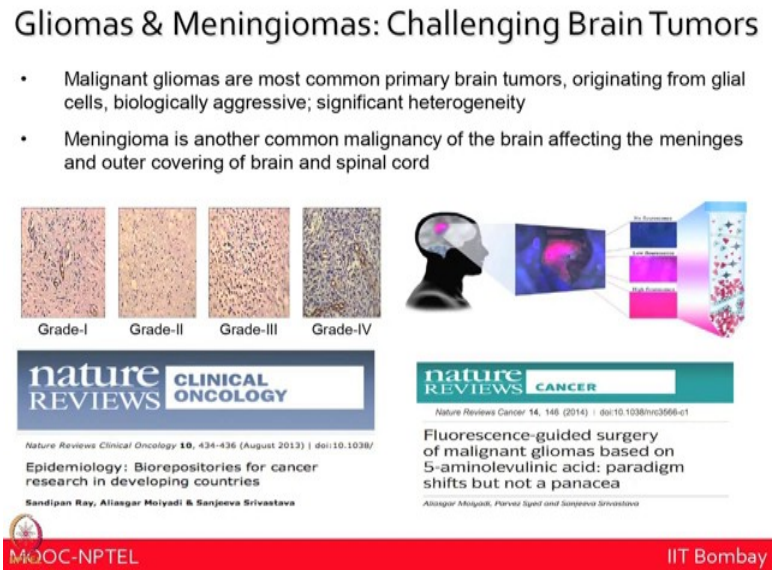


There has been extensive genomics which has happened in this you know especially the glioblastoma multiforme brain tumor, but not much of the overall proteomics or the proteogenomics investigation has happened so far for various type of you know the low grade gliomas and meningioma and the other type of brain tumors. So, on one hand we definitely need good repositories or bio-banks for the bio specimen to do this kind of research. Additionally there are lot of challenges of the heterogeneity of these tumors which we have described in some of these you know review articles, which showed the challenges of doing this kind of research, because any large number of sample and if you have large number of samples, the patient will be affected from you know the variety of

you know issues. And therefore, so much heterogeneity is there even from the same patient.

So, to investigate that you need of course, complementary approaches, you need large number of samples, you need very robust data analysis work flow; and together only, you can try to obtain some information. So, let us say how we can use the proteomics work flow to address some of these problems. So, you can get variety of sample type, you know either from tissue, serum or the cerebrospinal fluid.

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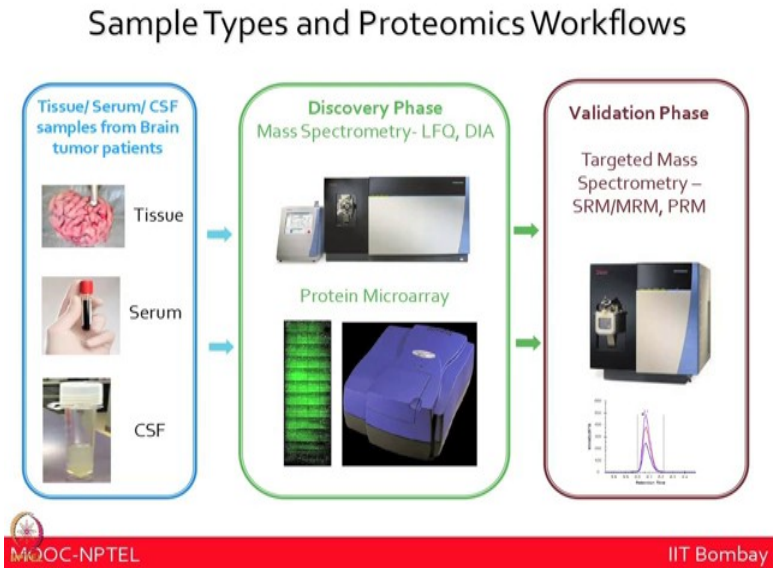


Now, use this sample type biological specimen to you do discovery either using mass spectrometry based work flow or protein microarray based work flow. And after identifying the potential candidate targets, then one could go to validate the biomarker using target proteomics based workflow. So, some other technology which we talked in my first lecture let us try to see how we can put them together and use them in this kind clinical problem. So, we wanted to ask a question whether this kind of you know proteomics investigation which we are trying to address for a brain tumor could identify some of the key networks and the potential targets for different grades of meningioma brain tumors. So, we are now looking at meningioma.

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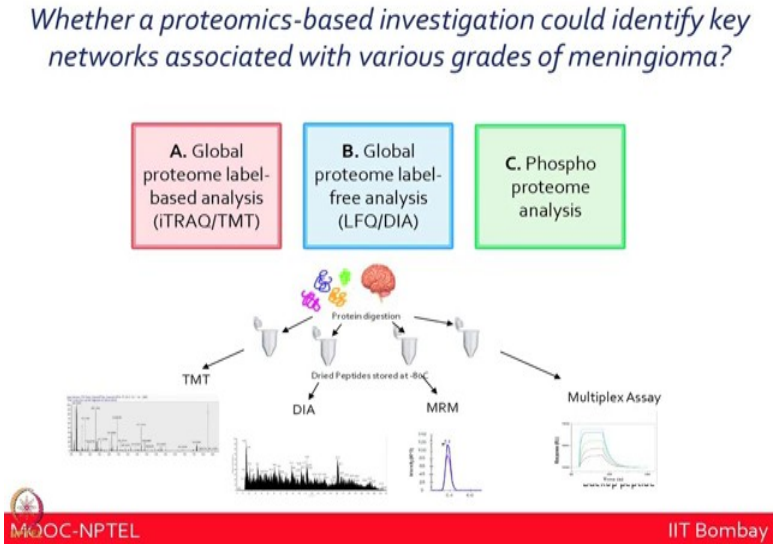


And then we are using three different type of complimentary approaches. One is global proteomics where aim is to look at all possible proteins and use their quantitation using either iTRAQ or TMT based work flow, or use the global proteomics work flow using the label free analysis which is LFQ - Label Free Quantification or DIA - Data Independent Acquisition, or we do the phospho-proteome analysis just to looked at the enriched phosphopeptide residues available from these peptides.

To do this let us say from the same patients, the brain tumor sample when it comes, we can do the protein extraction, and you know split those particular peptides after digestion into multiple tubes. And now each tube containing peptides could be utilized for either iTRAQ or TMT workflow, LFQ or DIA workflow, or you can use for eventually for the validation strategy, using you know SMR MRM assays or do some multiple multiplex assays in future.

So, now the same sample you prepare in sufficient amount which could be utilized for different type of strategies. So, let us go one by one. We use, we took the patients of meningioma, different grades of meningioma patients if we say grade 1, 2 and 3, 3 are very few. So, we had mainly grade 1 and grade 2; grade 2 are more of malignant patients. We use the iTRAQ based work strategy as well as TMT based workflows to try to compare different grades of these patients.

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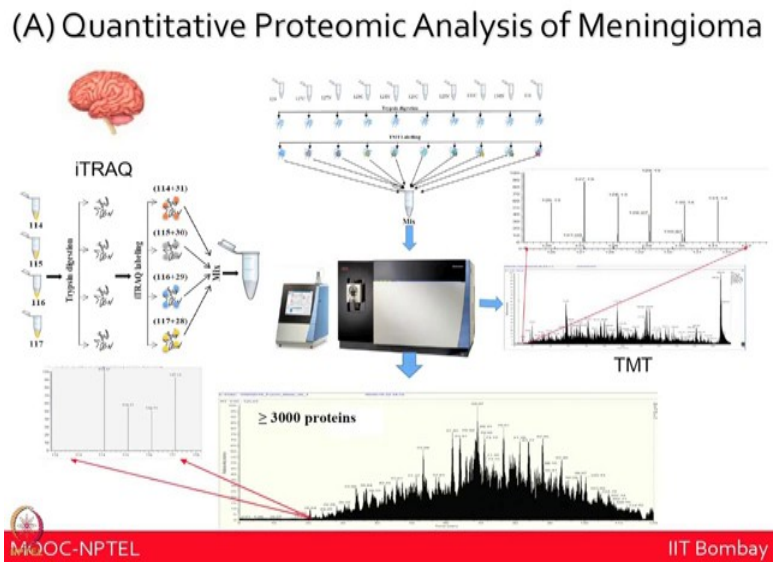


Reliably we could get almost 3000 proteins from the you know quantitative comparisons.

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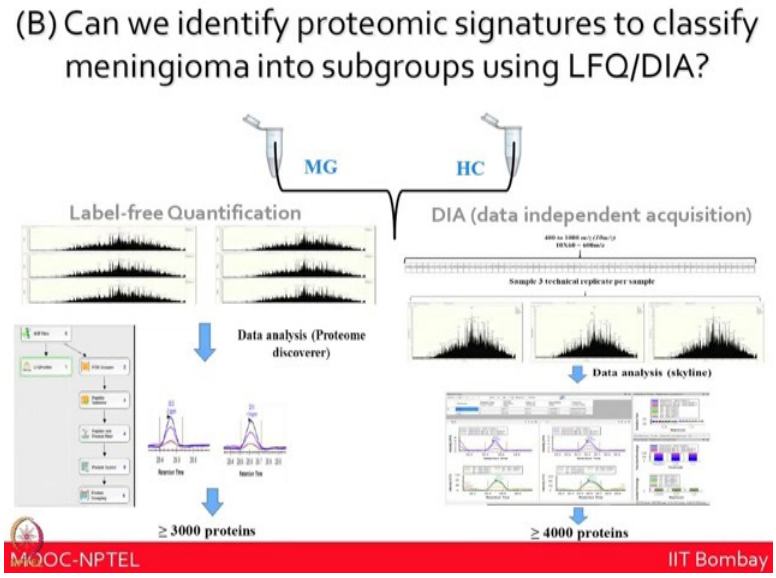


And then we also use a same samples to now look at label free quantification or data independent acquisition. Using DIA workflow, we could actually reach out now to almost more than 4000 proteins which is after much you know stringent screening that we can screen so many patients, so many protein from you know each patient which now we can quantifying the label free manner. So, same patient samples you are not trying to use either label based strategy for doing iTRAQ based quantification or you are using the

label free quantification to compare how the controls look different from the you know grade 1 patient or a grade 2 patient. Similarly, we try to enrich the phosphopeptides after passing through titanium dioxide column.

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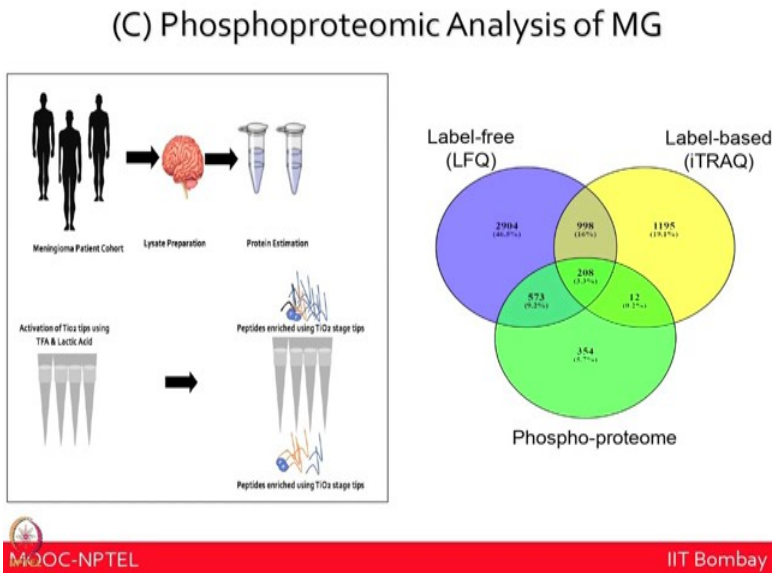


And then use this phospho enriched fraction for the analysis. And now we try to compare all the three information from the label free, iTRAQ and phosphoproteome by each of these provides you know some set of unique information. But what you will also curious to see which fraction is actually showing common pattern, because that shows that you know these are reproducible from different independent technologies as well as these are also showing us the trends for the phospho peptides or the PTM modification.

So, almost 208 proteins or you know sizable amount of stringent proteins showed a common pattern emerging from various independent technologies. Some of those we took forward. And now we try to look at the plotting in the heat map formation how that compared from the meningioma grade 1 and grade 2.

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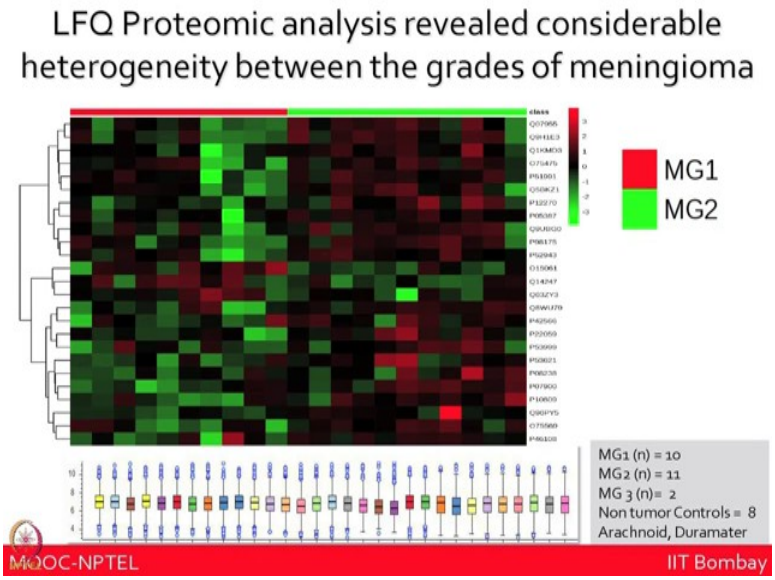


And you know as you can see the green line here, this part is for the meningioma grade two which looks quite homogeneous, whereas, the red line shows the heat map for the meningioma grade 1 patients. And this shows that you know there is lot of heterogeneity in the grade 1 patient not all grade patient look exactly same, a set of patients look slightly different than the other set of patients.

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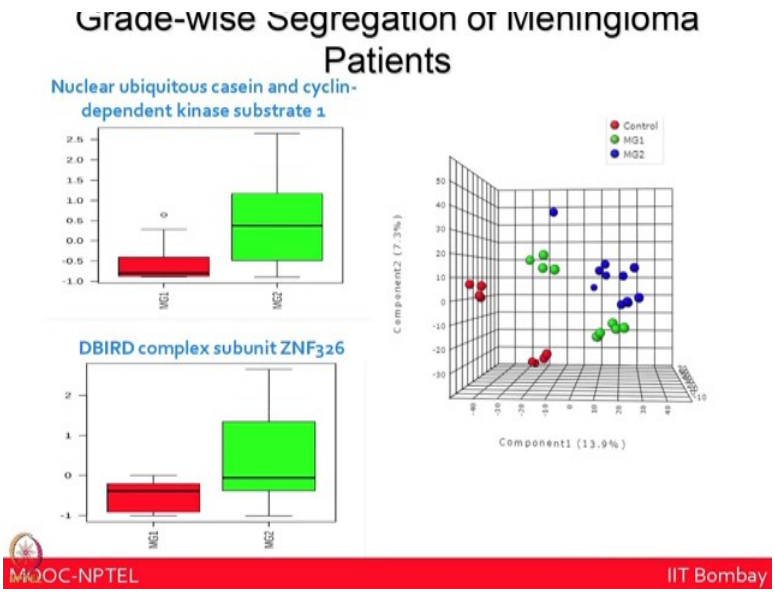


When we try to plot the PCA plots to look at the pattern based on these proteins can we now segregate the patient population, it was interesting that these are the two types of

controls dura mater and arachnoid. They are actually different from the different origin of the brain. So, they look different here. The grade 1 patient look like they are forming two subtypes, and then grade 2 they are quite homogeneous they look this you know very much together. And based on this information, we are now finding some you know interesting protein leads and kinases which could be taken forward.

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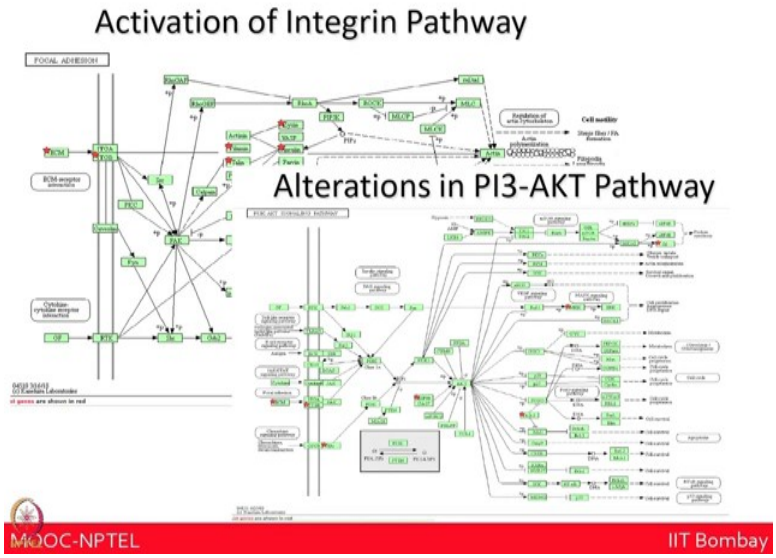

But, what is most interesting from these kind of proteomics and you know big omics studies, you get to see large number of changes and you try to put them together and analyze based on the pathway analysis that most of the changes are perturbing which type of network and pathways. So, looking at this information, we found that Integrin pathway as well as the PI3K-AKT pathway where greatly affected because of the meningioma disease. So, now, the logical flow was to try to investigate this problem, and look at this pathway much more detail.

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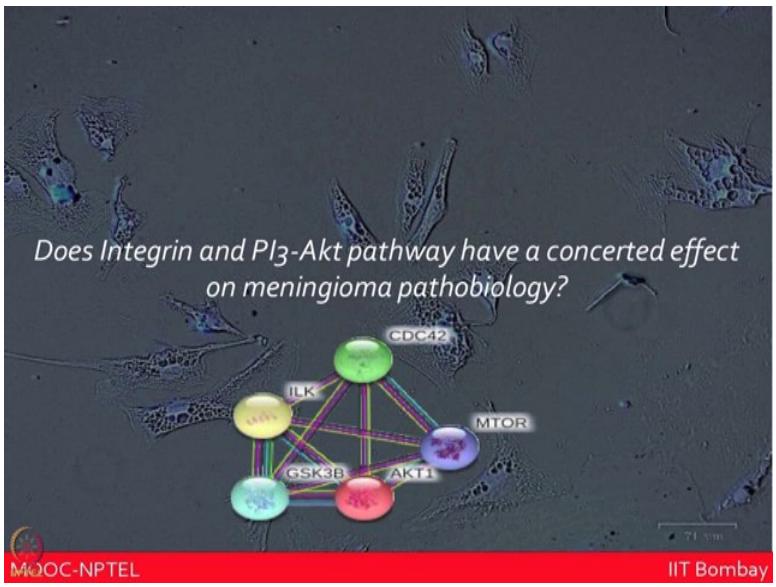


Many of the you know the in network this is what is shown here these proteins were identified from our you know discovery workflows. So, our question was whether integrin and PI3K-AKT pathway have some concerted effect on the meningioma pathobiology.

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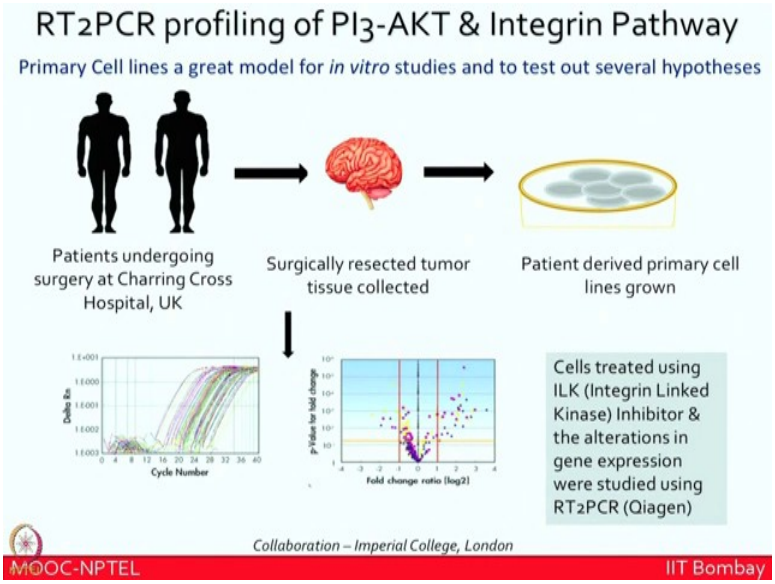


To know this in the collaboration from you know Dr. Nell's lab from Imperial College, London; one of my student she went and look at the patient derived cell lining of meningioma. And then treated them with one of the inhibitor which is ILK - Integrin Linked Kinases to look at what is the effect of this particular inhibitor on these meningioma patient cell lines. Because our aim was to look at some of the targets of the PI3K-AKT pathway, and whether this inhibitor could actually block or affect some of these proteins.

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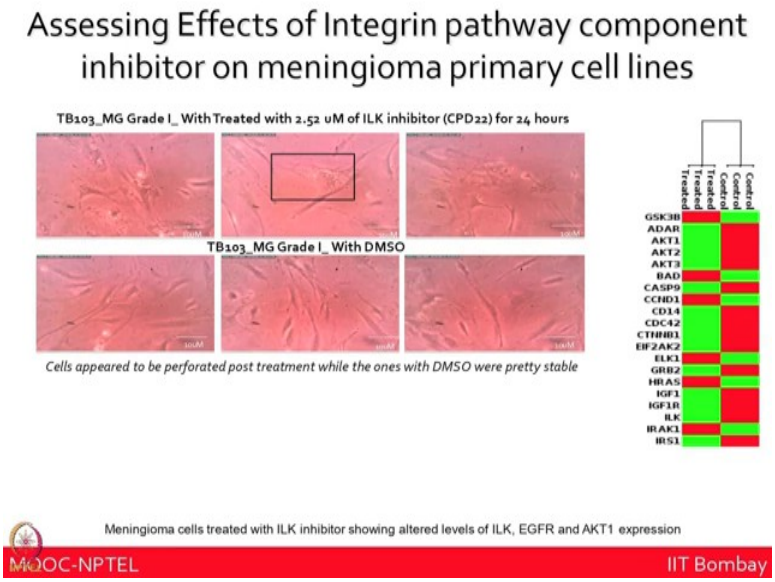

And interestingly it showed that you know this inhibitor had the effect where it could actually you know potentially affect the targets which we have talked in the network analysis. And then after doing the real time PCR analysis, some of these targets are again confirmed that you know this particular inhibitor has perturb these genes of interest. Further studies are still undergoing and you know currently you know under way to do more of the biological replicates, but the promise here is that if you can get these kind of therapies, this could be a surrogate way of you know adjuvant therapy, where if you cannot treat the patients you know with the surgery can you use some inhibitors to at least try to control the tumor for some time. Additionally, the clinicians give us some sort of you know focus questions to address especially based on the radiological observation.

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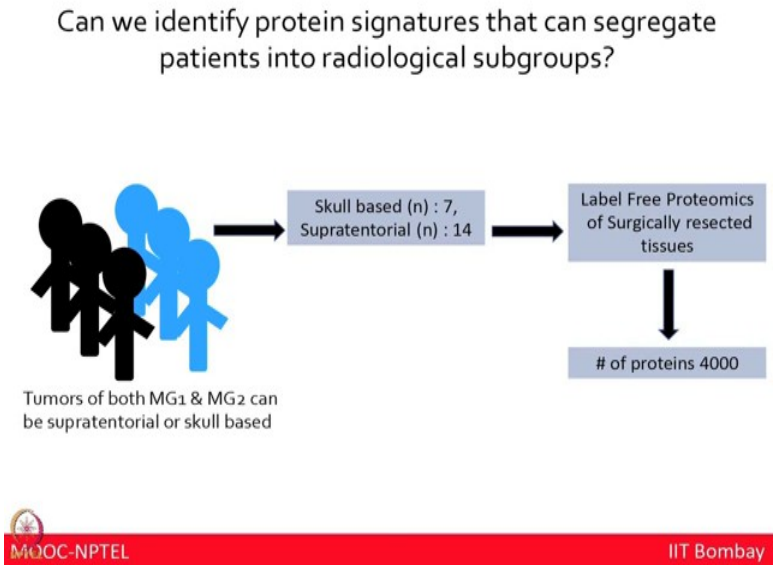


Whether the skull based or supratentorial locations of the brain may have an effect at the proteome level.

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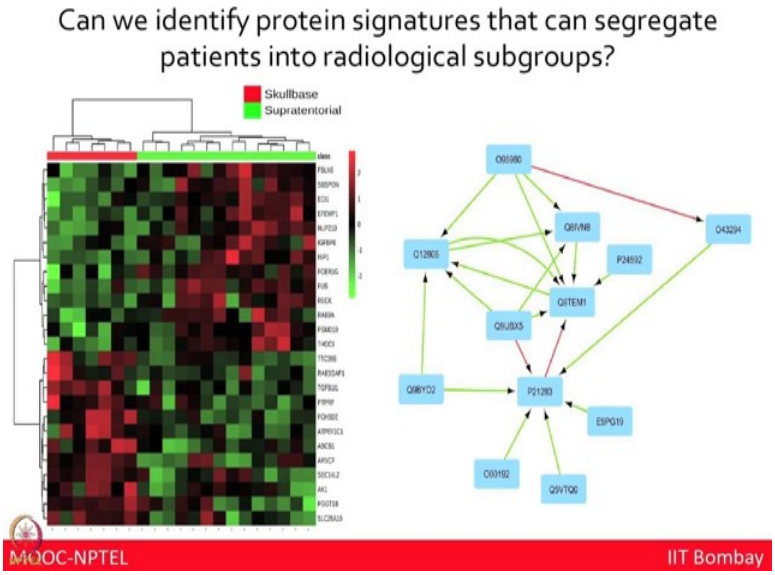


To do this now if you have done the proteomics within label free, we can now use a same data and now just you know try to analyze that in different manner. So, in this case now we try to look at how the skull based or supratentorial you know brain tumors are actually getting segregated you know in the meningioma population.

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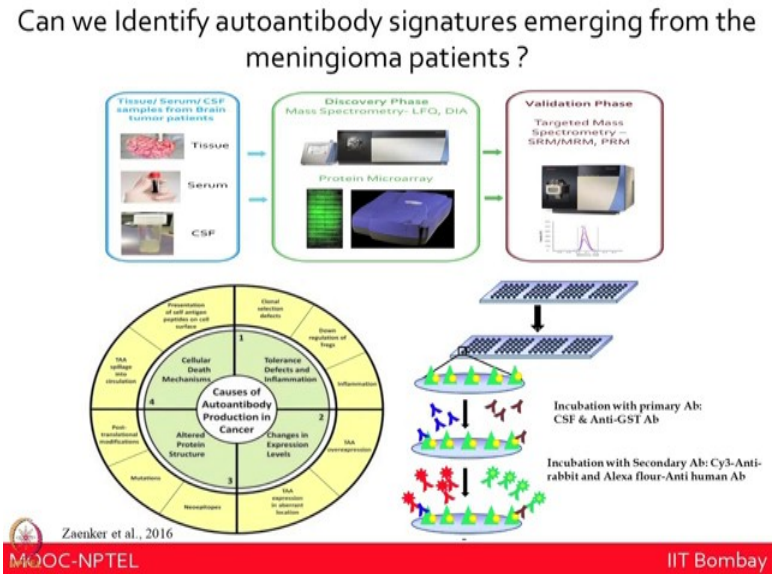

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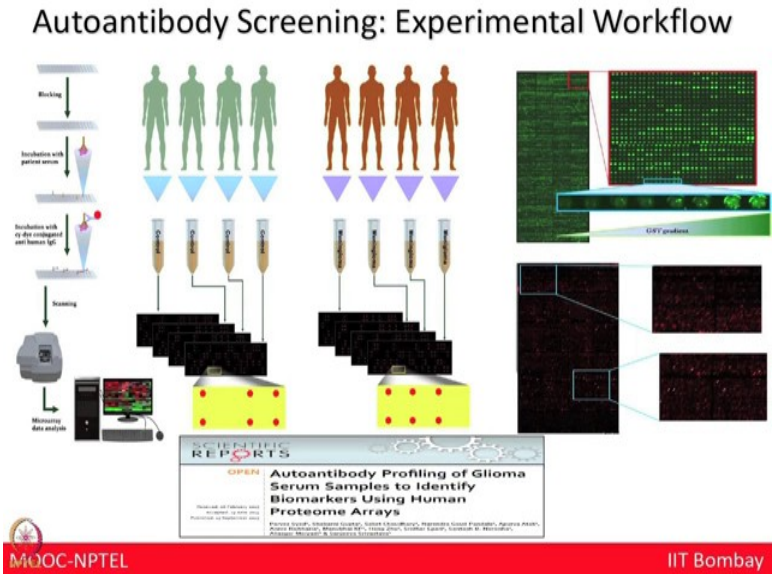


And very interestingly it looks like they are very clear segregation of these two type of subtype based on the radiological observation. So, looks like radiology is much more closer to the molecular signatures as compared to pathological observations, but of course, some of these we need to still take forward with more validation. But what is coming out interestingly after looking at these data and doing big data analysis from the artificial neural network, we could now see the impact of some of the positive regulators and negative regulators, and how they are you know going to affect these type of brain tumors.

So, this is where you can see that you know how you can start from clinical problem of interest identify the targets and then do big data analysis to try to get some sort of meaningful conclusion out of this information. Additionally in a you know rather than using mass spectrometry alone, we also try to utilize the complementary technology or protein microarrays, to add this question can be identified autoantibody signatures in meningioma patients.

And goal here was to use protein arrays platform take patients clinical sample, serum sample, probe them on the chip, and if there is any antibody generated in the patient sample they will bind on the chip which is having all the protein antigens printed. To do this work we did collaboration from Johns Hopkins where we have all these you know almost 19000 proteins printed on the chip.

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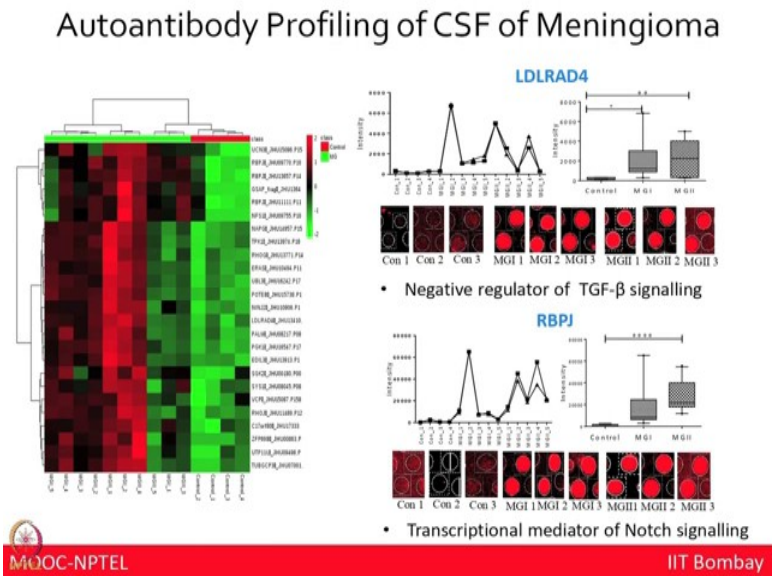


Now, if we see a signal from auto antibody which is showed in the next slide this looks like you know these are the control samples of the individual meningioma grade 1, meningioma grade 2 patients, and some proteins are showing you know very strong response and this could be potential auto antibody biomarkers for the detection of this particular tumor.

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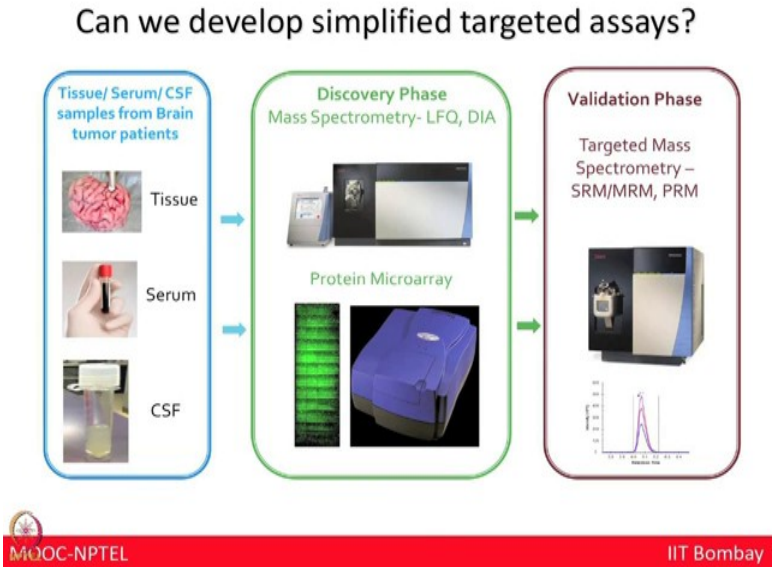

So, coming back to the various workflows which one could utilize, we have used both mass spectrometry and protein microarray based work flow. Then there is a need to do validation.

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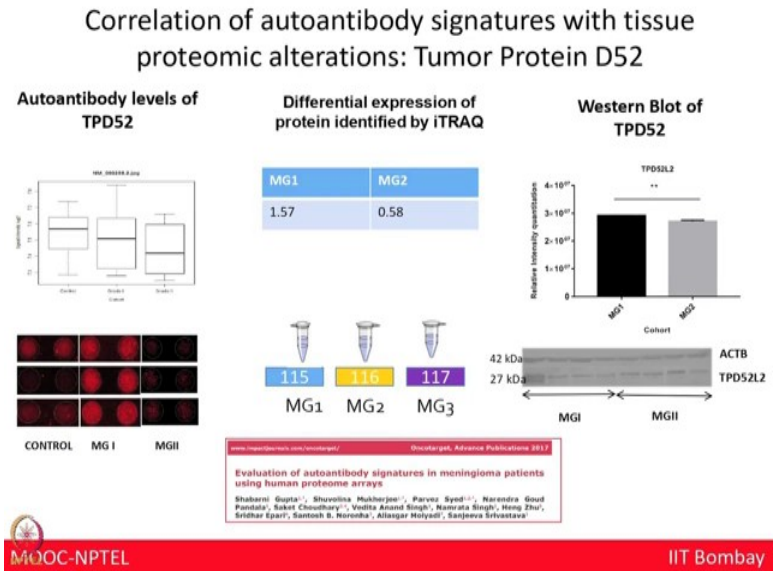


And for validation you should you know not limit yourself to respected technology, rather use whatever is available to you ELISA, western blot or microarrays or even looking at the you know targeted based workflows to get more confidence. And many times you will see that you know why antibodies are not able to detect and make lot of changes because they might have been raised for a given epitope. But your other type of complementary technologies are showing you much more higher changes and therefore, then you can get gain more confidence by having multiple technologies to validate the proteins of interest.

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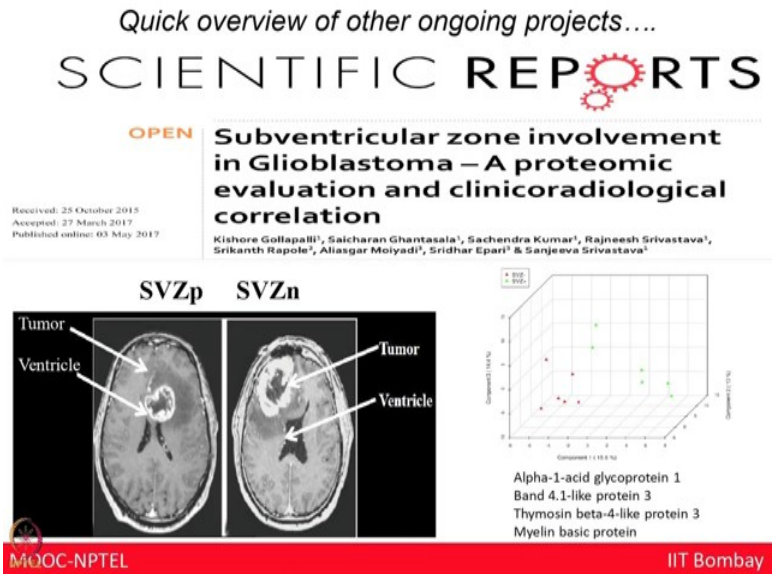


We have also tried to address a specific questions from the clinicians especially in the gliblastoma multiforme patient, looking at the location of the brain tumors, now what could be the impact of you know the tumor location to the ventricle region is a very close or is it far off, these patients will be you know surviving less, this will survive longer, it is much more aggressive tumors here known as SVZ positive or SVZ negative, and can we look at the proteomics signature to try to classify these kind of tumors.

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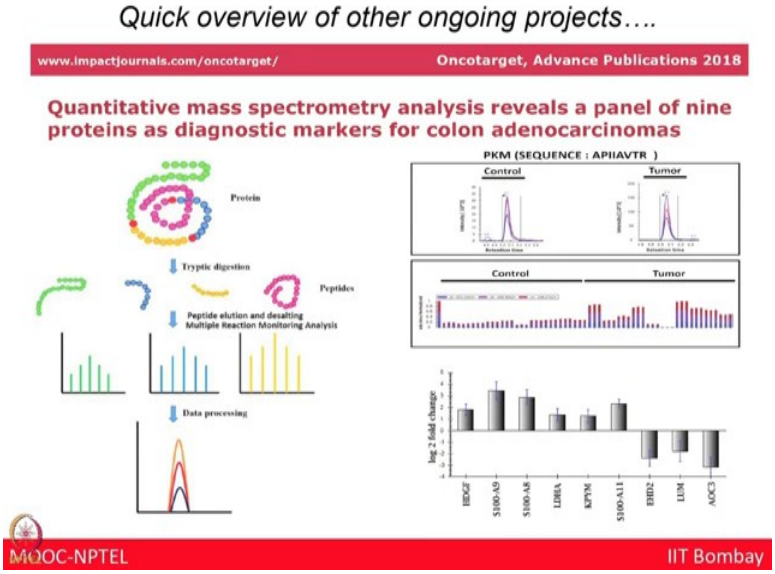

In another project in collaboration from you know Prof. Gil Ast Lab, Tel Aviv University in Israel, we try to investigate the colorectal cancer problem colon adenocarcinomas, and we found the panel of 9 proteins looked very reproducible across large number of patients affected from the colon cancer in the middle east countries. And we are not trying to validate this in also Indian patient samples.

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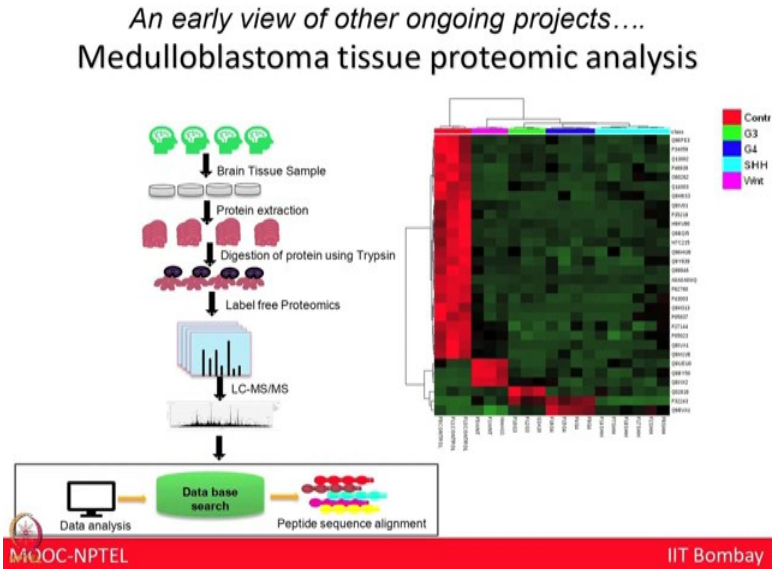


Another interesting problem is medulloblastoma the pediatric brain tumors. Again we are trying to provide the proteomic based subtype signatures from the various type of you know the patients affected from there you know the Wnt type or the SHH type or group 3 and group 4, what could be the possible protein alteration in these type of you know the children these pediatric brain tumor population.

Finally, we are trying to look at variety of approaches of proteomics. Also we are trying to use the various you know basic science tools of you know doing the transfection, and you know understanding the effect of these you know mutations on these particular tumor type. We are developing various type of clinical assays in collaboration from the industrial partner of targeted based proteomics.

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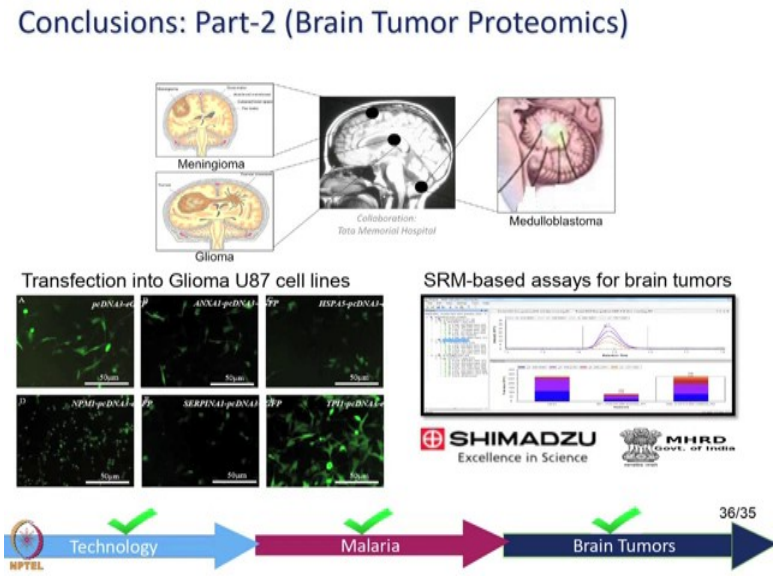


So, overall I try to give you the glimpse of how to put together proteomic technologies in context of various clinical problem.

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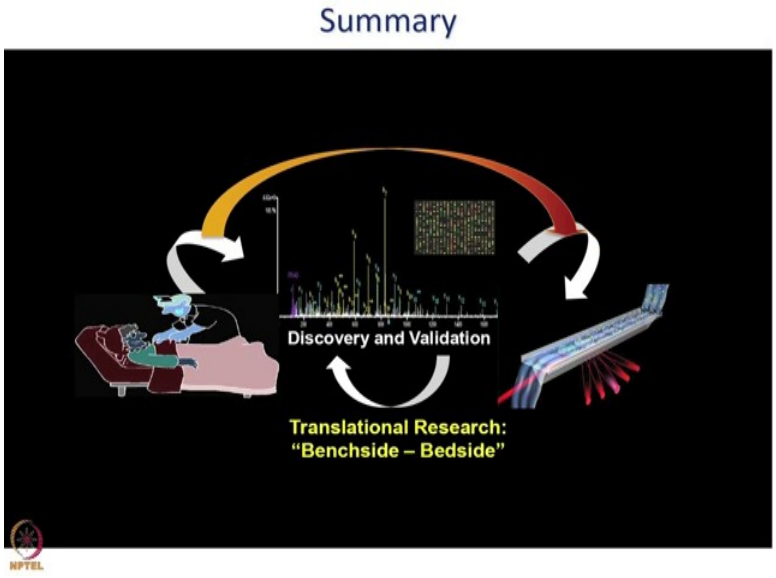


We try to address two clinical problems of looking at the severity of infection in the case of malaria or also looking at what could be the possible queues and subtype molecular classification of brain tumors. So, eventually these kind of technologies may provide you and give you the possible targets which could be translational potential to take the lead from the bench side to the bed side, and that is what is the goal for many of our you

know labs working in the areas of genomics and proteomics, but how not to leverage this information and integrate that as a part of proteogenomics, this is what this workshop and course is about. We would like to utilize the proteogenomics information for the better patient care.

Thank you.

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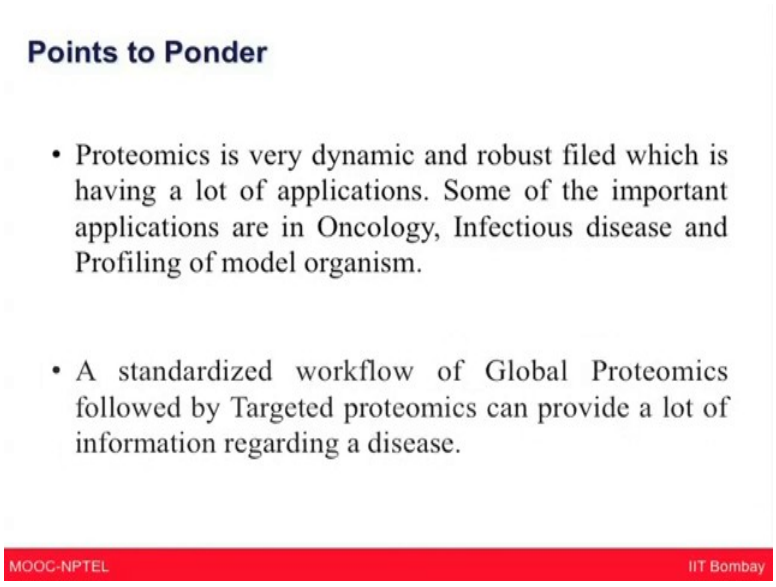


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