Proteomics : Principles And Techniques

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Lecture No. # 19
Applications of 2-DE and DIGE

Welcome to the proteomics course. In today's lecture, we will talk about applications of 2 dimensional electrophoresis, and difference in gel electrophoresis. If you recall in the previous lecture, we started discussing about some case studies about 2 dimensional electrophoresis applications. So, as you have studied in this module, what are different type of workflow involved in performing 2 dimensional electrophoresis experiments? We have gone through a step by step, starting from the protein extraction, doing the quantification, isoelectric focussing, which separates protein in their first dimension followed by doing the equilibration, preparing the strips for second dimension separation, and then you separate the proteins on the s d s page based on molecular weight, followed by stain the gels to see the visualise spot, and then scan those images analyse that by using various software, and then perform some statistical analysis to obtain some biological insight.

Now, this work flow remains same, regardless of whatever application one want to use in their different type of biological questions. I started discussing about 2 dimensional electrophoresis applications in the last class, I gave you an overview of the 2 d e at that time. So, let us continue our today's lecture from the same theme, and let us discuss some more case studies on how people have employed 2 dimensional electrophoresis, the power of this technique to resolve thousands of proteins, and compare those for various differential proteomic applications.

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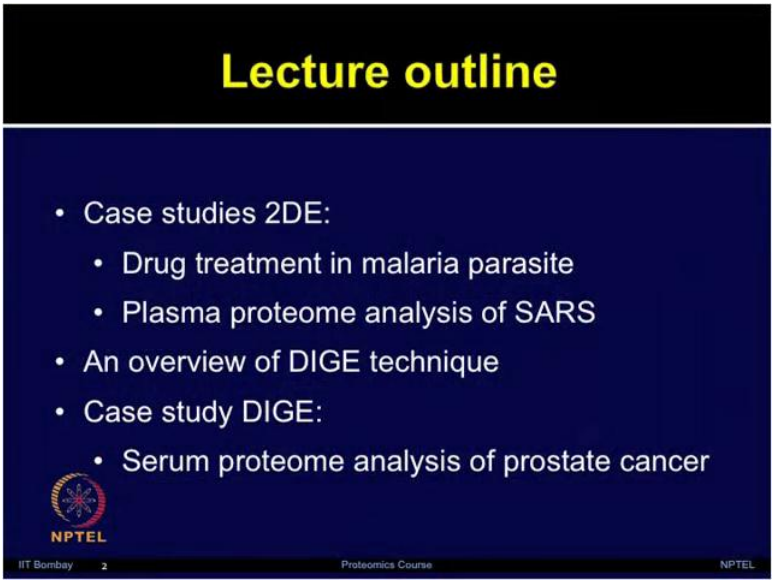

So, in today's lecture outline first we will continue our case studies on conventional 2 dimensional electrophoresis, we will talk about study based on the drug treatment on malaria parasite, plasmodium falciparum, till then take a study on the plasma proteome analysis of SARS virus. Now, after looking through these studies and discussing about these case studies, then we will move into the difference in gel electrophoresis. Again if you remember, we have studied that in much detail earlier, today I will give you again just an overview to refresh you about the DIGE technique, and then we will see one application of DIGE technology, how it can be used for serum proteome analysis of the prostate cancer patients.

So, what I want to convey that there are multiple applications one can actually perform by using this platform, and just to overcome certain limitations which are inherent for the conventional 2DE.Now, people are also employing the 2D-DIGE approach, but the work flow almost remain the same except the sample preparation part, and then this powerful technology can be employed for several applications.

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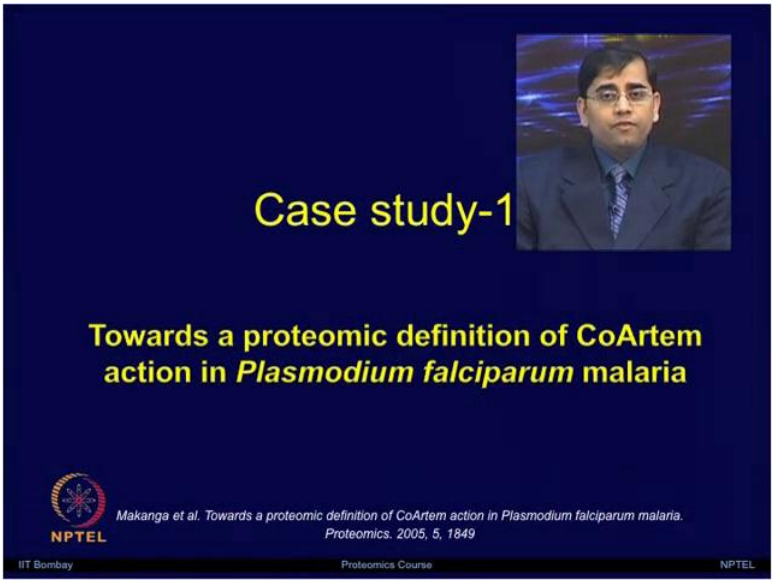

So, let us start with the first case study towards a proteomic definition of coartem action in plasmodium falciparum malaria, a study by Makangaet al in 2005. So, as you know each year hundreds of, millions of new malaria infection cases result in over one million death worldwide, but due to the lack of effective vaccine and wide spread resistance to the anti malarial drugs is still lot of deaths are happening, and the malaria problem is still posing challenges for its control.

The anti malaria therapy of chloroquine and proguanil theme mean, these have not been able to control the mortality rate, because of the antimalarial drug resistance development. So therefore, there is urgent need for identifying new dot targets, as well as understanding the codes of the action of these drugs by applying various type of high throughput techniques.

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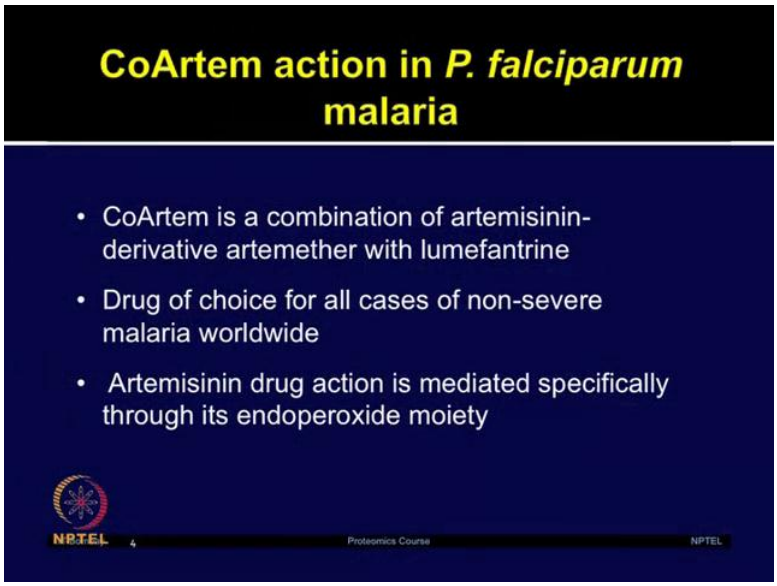

So, in this paper authors have discussed howtwo different drugs, which are effective for the anti malarials can be study for looking at the proteome changes in the plasmodium falciparum parasite, So,CoArtem is a combination of artemisinin derived artemether with lumefantrine.

How, these two drugs behave, and how the proteome changes occur due to the action of these two drugs were studied in this paper. So, that is applied proteomic approaches, the 2 dimensional electrophoresis to study the proteomic alteration of each of these drugs. So, these drugs are applied as a drug of choice for all cases of non severe malaria worldwide, the artemesinin drug action is mediated specifically through its endoperoxide moiety. However, the more detailed mechanism of action of these drugs are still unknown, so the purpose of the study was to investigate the action of two active components of new antimalarial CoAltem, r t method and lumefantrine on human malaria parasite plasmodium falciparum. An author's tried to look for the alterations in parasite proteome, which were induced by each of these drugs.

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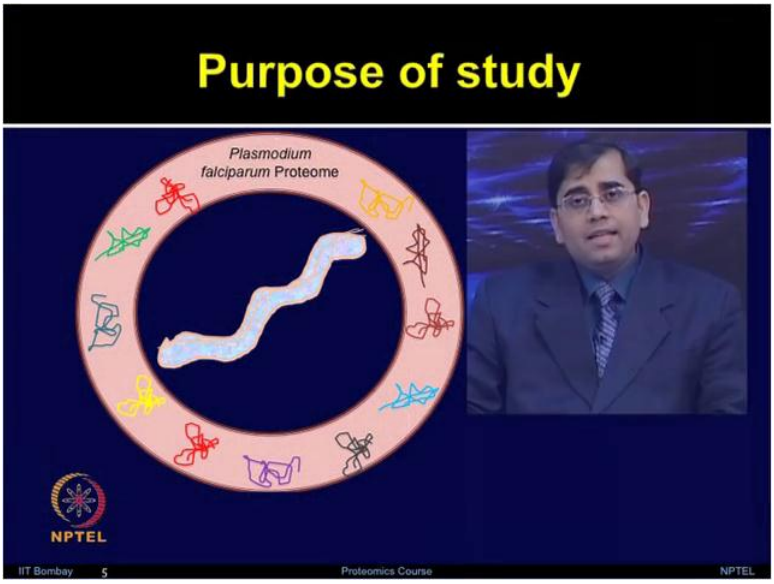

To obtain the insight of the proteomic alteration they separate the proteins on the 2 dimensional electrophoresis gels, and compare the response of these proteomic alteration based on the these two drugs. And then they identified certain proteins which were either commonly expressed due to these drugs, or they were differentially expressed due to the drugs. So, certain proteins were found to be commonly up regulated due to both of these drugs, and certain proteins have the different patterns.

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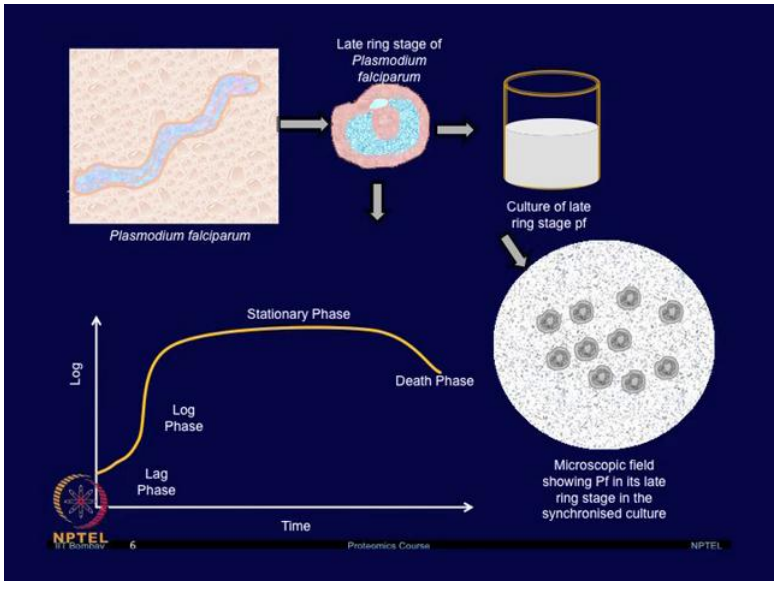

But, before looking at the proteomic alterations author's first determined ic 10, 20, 50 and ic 90 values for both the drugs, ARM and LUM, you will use the abbreviations now for artimesin in and lumefantrine. An effect ofthese concentration of drugs on parasite growth over 24 hours was characterised, as you can see the growth curve in this slide, so synchronised ring stage parasite cultures were harvested over 24 hours period after the exposure to the ARM and LUM. The parasite growth was determined by using hypoxanthine optic acid. After establishing the culture conditions and the drug concentration, then author's look for the proteomic alterations.

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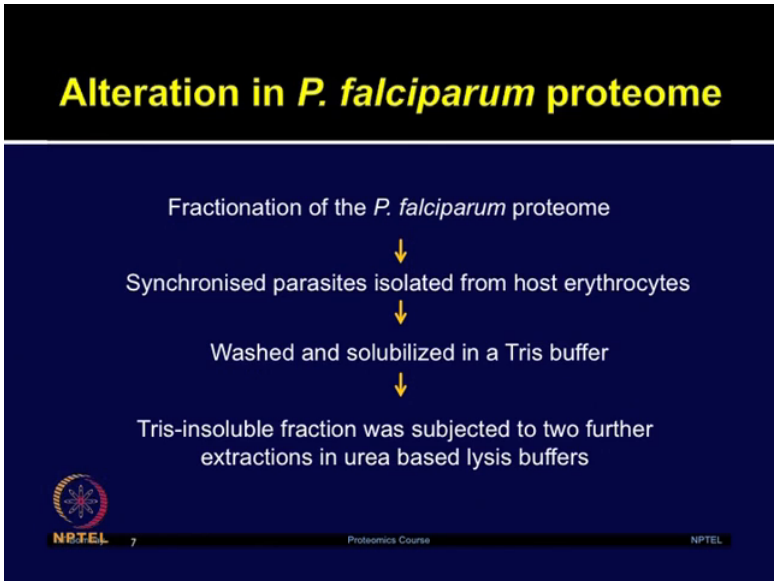

So, first of all they did the fractionation of the P falciparum proteome, synchronised parasites which were isolated from the host erythrocytes, wash those initially and then solubilise that in a tris buffer recipe, the tris insoluble fraction was further subjected to extraction in the urea based lysis buffer. Once protein extraction was done then author's used IPG strip of p H3 to 10 ranges for the first dimension separation of protein in the linear IPG strips.

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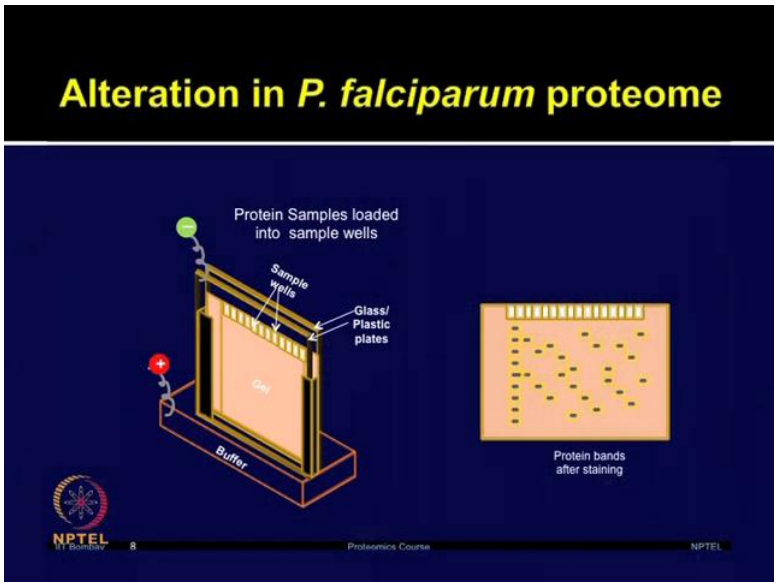

After the i e f was done they equilibrated these IPG strips, and then applied that on 1 2.5 percent of the vertical SDS gel. After the second dimension separation based on the molecular weight, then these gels were stained with the silver or coomassie brilliant blue stains. So, by employing two dimensional electrophoresis, and comparing the gel images by using the p d quest software, authors were able to see that there is a differential proteomic response which is drug specific.

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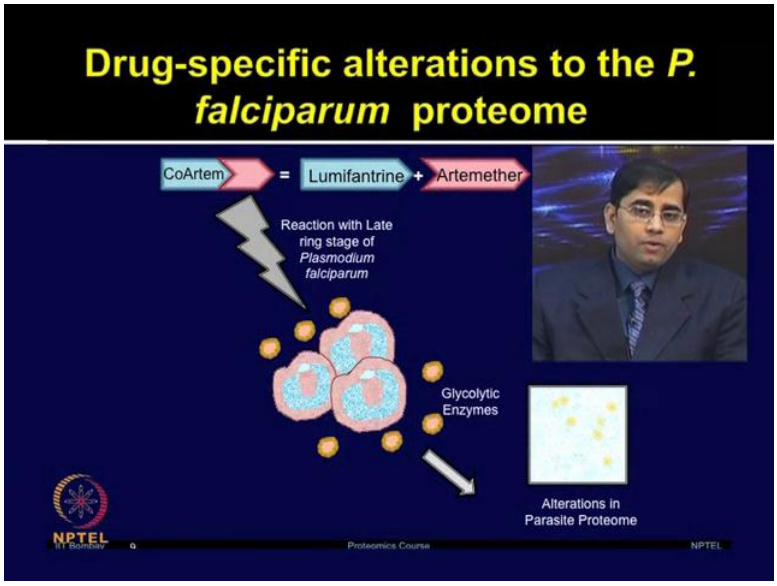

So, quantitative analysis of the altered protein expression levels following exposure to the ARM and LUM were analysed, and then those protein spots which were differentially expressed and statistical significant, were further subjected to the mass spectrometry based analysis. So, the comparative analysis of 2D gels from untreated, and drug treated parasite protein fractions provided direct and distinct alterations in parasite proteome, following artemether or lumefantrine drugs.

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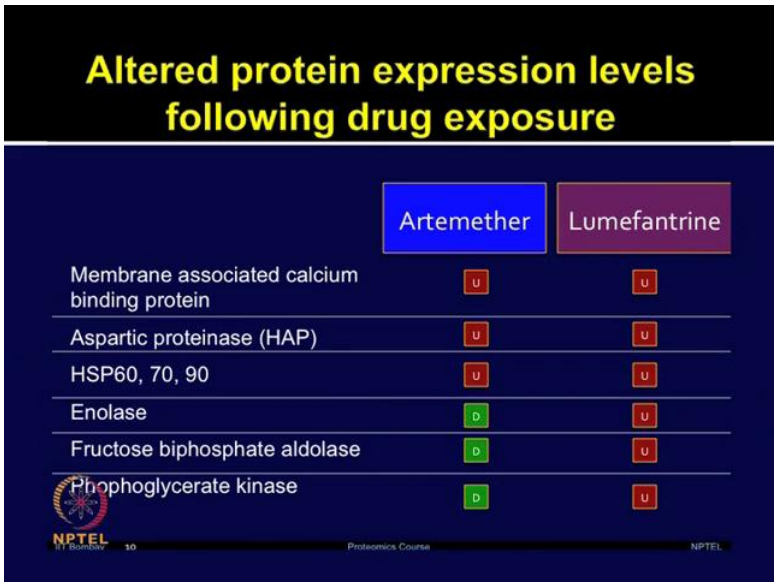

Certain proteins were identified, few of those showed common response due to both drugs. However, there are certain proteins which showed opposite trends due to each of these drugs. Proteins such as membrane associated calcium binding protein, was up regulated in both the drugs. Asparatic proteinase was also up regulated in both the cases. Heat shock proteins, such as HSP60, 70 and 90,those were up regulated due to both the drug treatments. Than certain proteins such as Enolase, Fructose biphosphate aldolase and Phosphoglycerate kinase these proteins were down regulated in artimether treatment, and up regulated in the lumefantrine treatment.

So, interestingly the ARM treatment resulted in the more than threefold down regulation of the glycolytic enzymes, such as Enolase, Phosphoglycerate kinase, Fructose biphosphate aldolase and glyceraldehydes three phosphate dehydrogenate.

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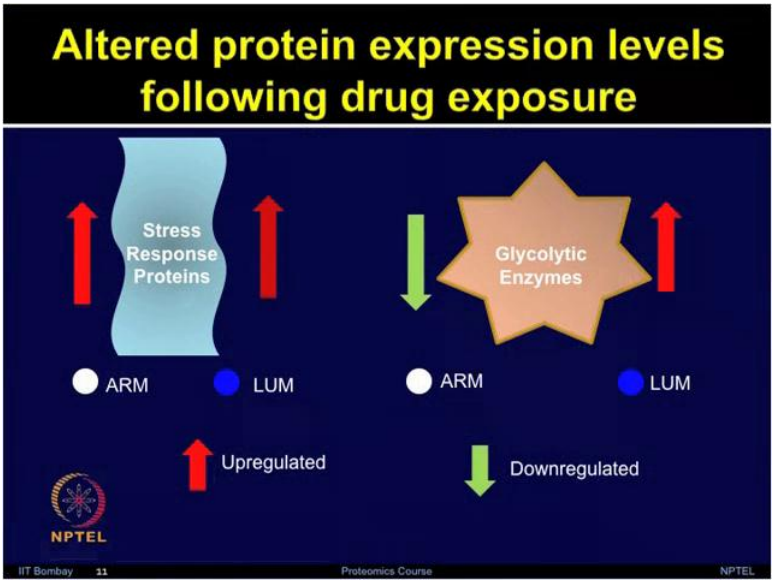

The expression of the same enzymes, were also upregulated more than threefold due to the lumefantrine treatment. However, the certain proteins such as the stress responsive proteins like heat shock proteins, which were commonly induced due to either of these drug treatments, which look like a general stress response as compared to very unique response to the given drugs.

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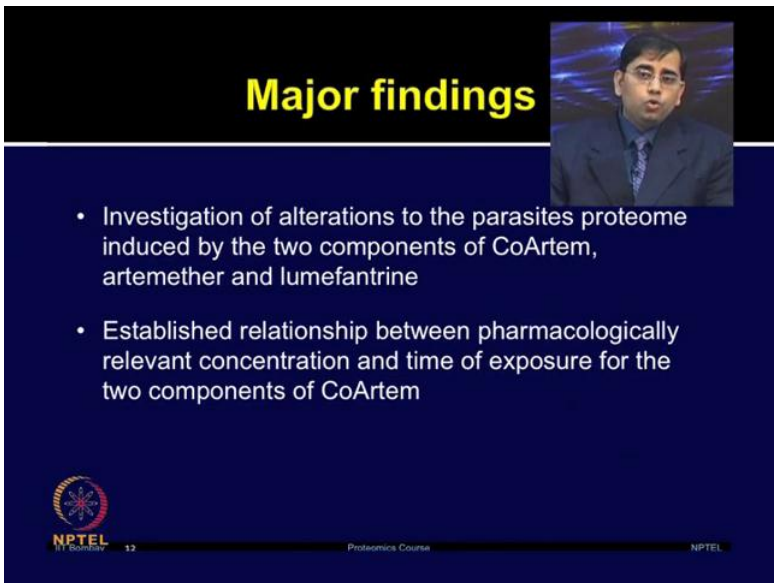

So, from this study the major findings were, the thought authors successfully investigated alterations of parasite proteome induced by two component of CoArtem, artemether and

lumefantrine. By using proteomic approach they investigated specific and nonspecific effects of two anti malarial drugs in pharmacological relevant conditions, expression of certain proteins were quite interesting including a membrane bound calcium binding protein, which was upregulated due to artemether and lumefantrine treatment. The study also established a relationship between the pharmacologically relevant concentration, and time of exposure for the two components of CoArtem.

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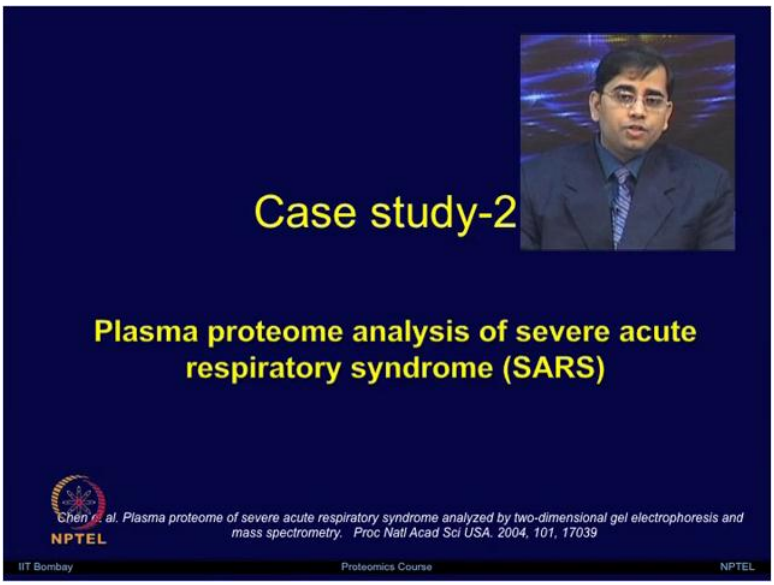

Let us now move on to case study 2. Plasma proteome analysis of severe acute respiratory syndrome SARS. Study by Chen et al in2004.

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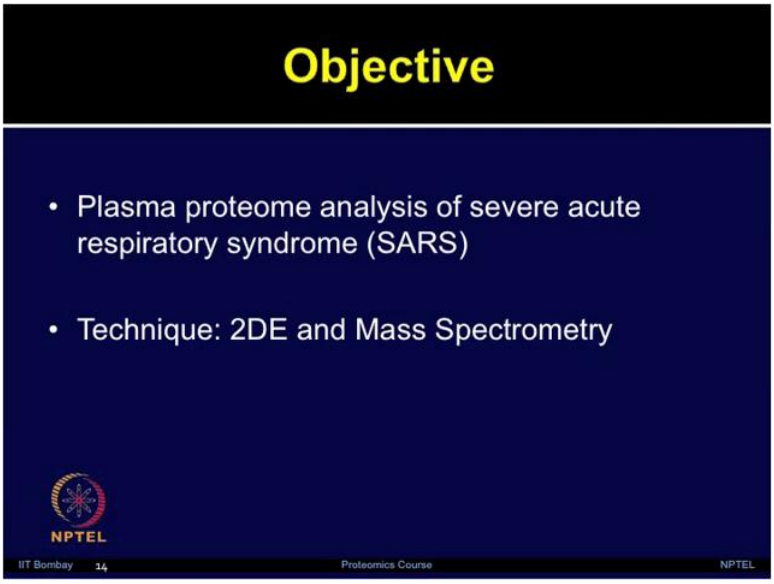

so the purpose of the study was to perform a comprehensive plasma proteome analysis of severe acute respiratory syndrome, and its comparison with the healthy individuals. In this study, authors employed conventional two dimensional electrophoresis analysed those images from the treatment with the controls, and identified the differentially expressed protein by using mass spectrometry techniques including Maldi-TOF-TOF and LC MS/MS. Finally, the interesting identified proteins were validated from techniques such as western blots. So, if you remember few years ago the severe acute respiratory syndrome occurred in 2002, 2003 and thousands of deaths were reported in several countries around the globe.

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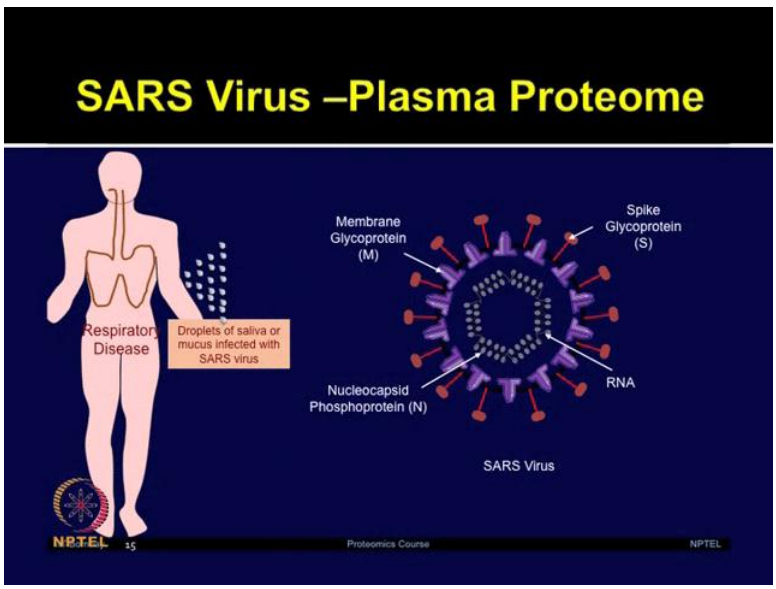

The genome sequence of SARS virus was already known, and the structure of main proteus and receptors were also known. However, the pathogenesis of SARS was not very clearly understood. Since serum or plasma, they provide a very valuable sample to identify the targets for diagnostic, prognostic and therapeutics. So, authors used plasma sample for the analysis of proteomic alteration in the SARS patients .For the plasma proteomic analysis, authors explore the possible pathogentic mechanism of progression of SARS by analyzing plasma proteins of 22 different plasma samples, which were obtained from the 4 SARS patient and 6 healthy controls.

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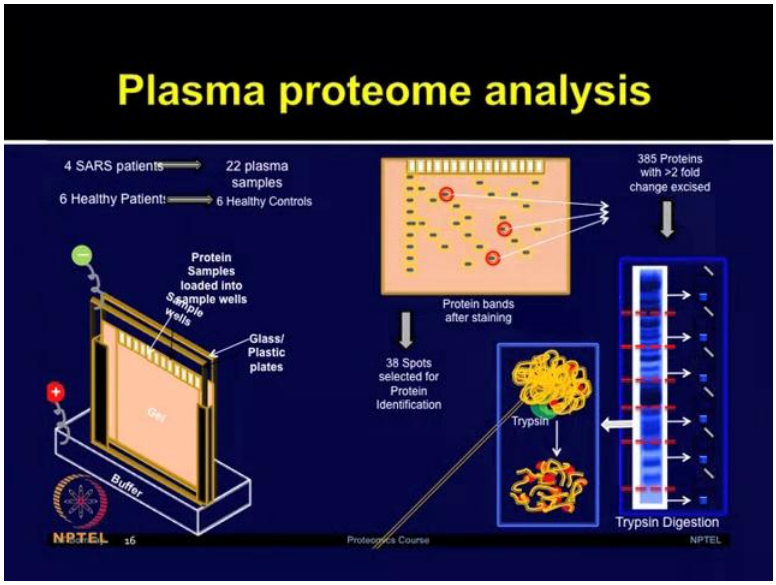

Authors analyzed plasma proteome by using 2 dimensional electrophoresis on 4 to 7 p H immobilized p H graded strips, and they stained the gels with a sensitive stain sypro ruby. So, the slide you can see the steps involved in the traditional two dimensional electrophoresis followed by the mass spectrometry, both the controls and the treatments, the healthy individuals, as well as patient suffering from SARS, the plasma was separated and then proteins were resolved on the 2D gels, differentially expressed proteins were further subjected to the in-gel Trypsin Digestion followed by the mass spectrometry.

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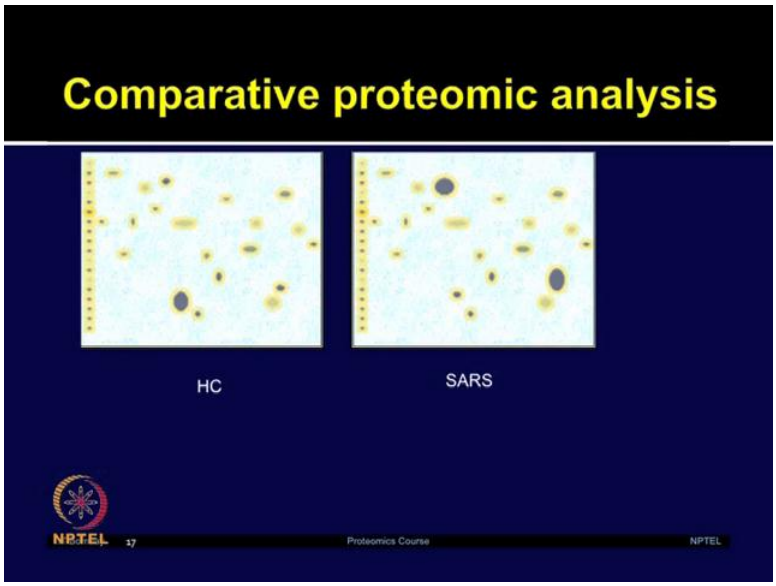

So, the comparative proteomic analysis of these 2D gels revealed that 38 protein spots were differentially expressed more than two fold change, and out of these spots there were 30 proteins which were up regulated, 35 were up regulated and 3 were down regulated.

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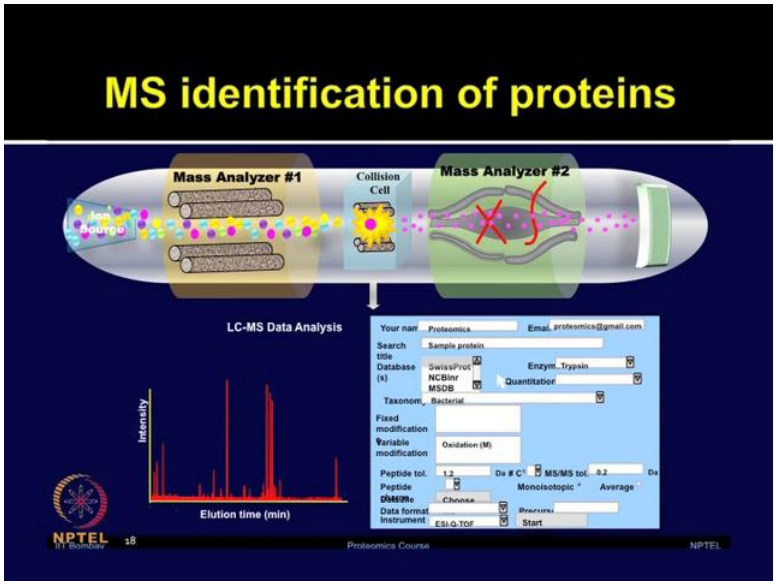

Now, these interesting 38, differentially and significantly expressed proteins were excised from the gels, and subjective to the Maldi TOF mass spectrometry for analysis.

Authors also employed liquid chromatography tandem MS/MS, system for analysis of these spots, and then the data was analyzed by using mascot search engine.

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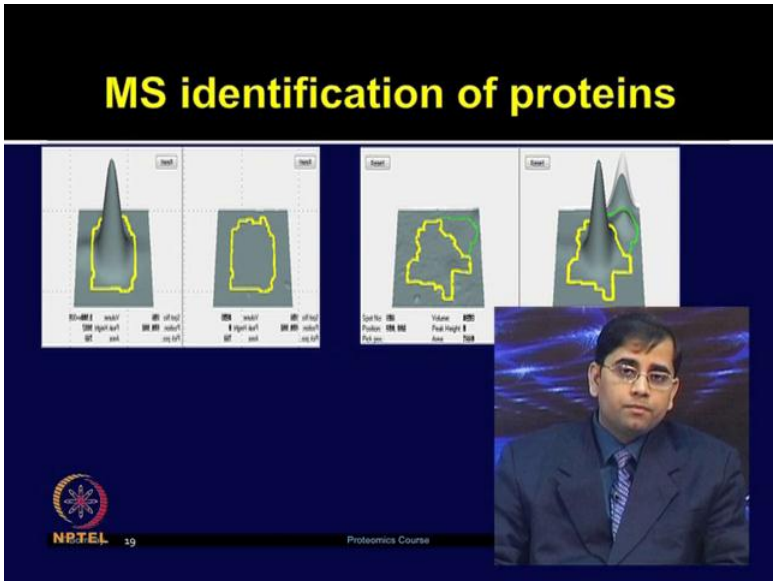

Now, after the identity of these proteins were established, the several proteins which were not detectable in the healthy controls and only observed in the SARS patient. The identities of those are included glutathione peroxidase P r x 2, retinol binding protein, vitamin d binding protein and serum amyloidal proteins. They also found that there are 8 proteins which were over expressed, and those included pigment epithelium derived factor, 2 h s glycoprotein, compliment factor H related protein and lucien ritsu glycoprotein. For complete list of the proteins identified, you can refer to the this manuscript, these are some of the interesting proteins which authors identified.

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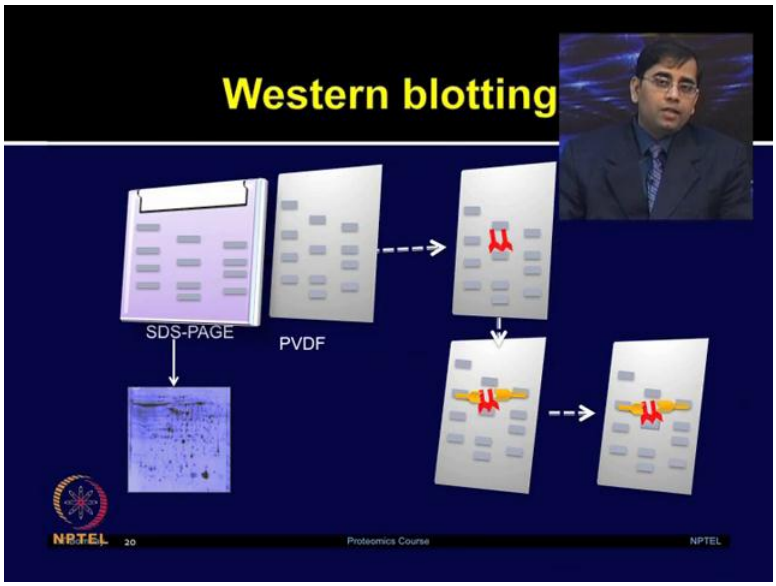

After the identity of these proteins established, then western blot analysis was used to validate few targets.(( )) identified proteins, author found that peroxiredoxin 2 was very interesting. So, they observed that an intracellular protein P r x 2 exclusively found in the plasma of SARS patients, but that was absent in the healthy individuals. These results demonstrated that P r x 2 can be present in the monomeric or dimeric form.

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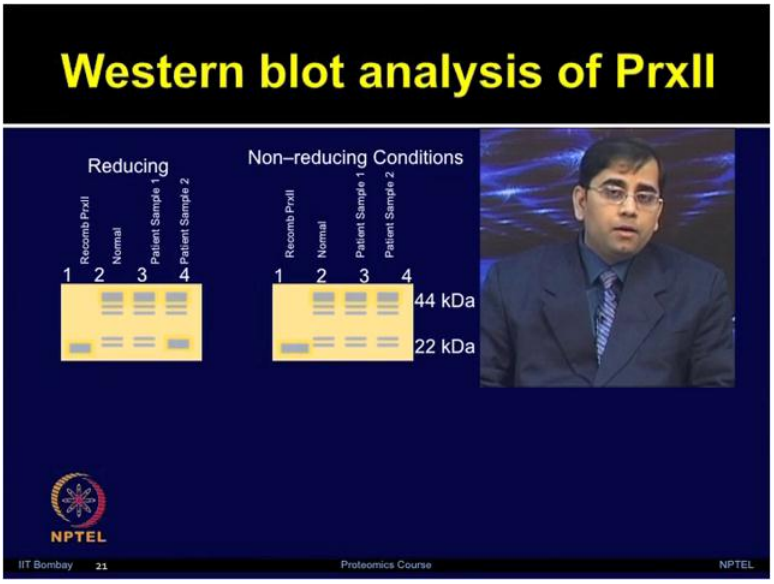

So, they performed the western blot in both, reducing and the non reducing conditions. As you can see in the slide, and then they loaded different samples including

recombinant peroxiredoxin, normal or healthy individual, patient sample one and patient sample two.

So, the western blot result shown that the P r x 2 is present in the monomeric form at the 22 kilo dalton, as you can see the molecular weight mentioned in the slide in the reducing condition, and in the dimeric form at 44 kilo dalton under the non reducing conditions. These result demonstrated that 4 out of 20 SARS probable cases, and four of the 20 SARS suspected cases showed higher upregulation of the plasma peroxiredoxin 2.

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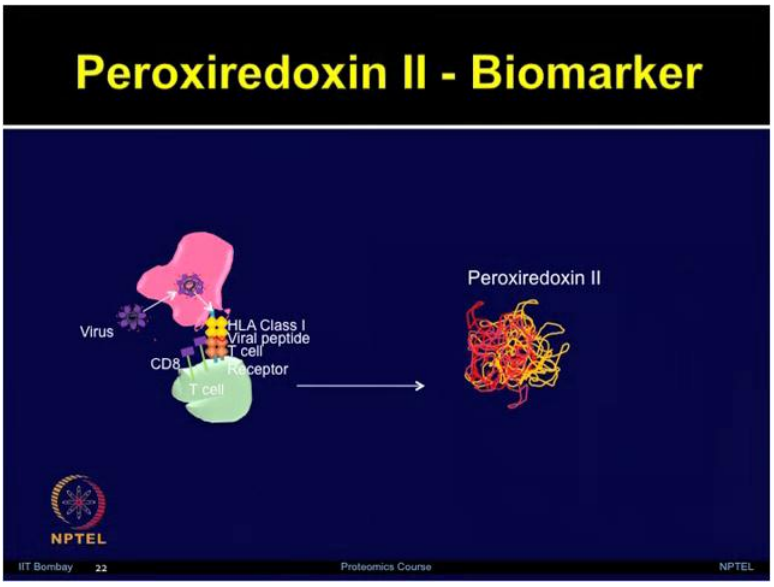

So, among various proteins which were interesting identified from this study, a peroxiredoxin 2 was appear quiet interesting, and also because it was validated by the independent techniques. So, the little of plasma peroxiredoxin 2 in patients with SARS was significantly high, proteomic analysis and western blot validation suggested that peroxiredoxin 2 may be used as one of the SARS disease associated biomarker. They also found there are several acute phase proteins or app's, those were differentially expressed.

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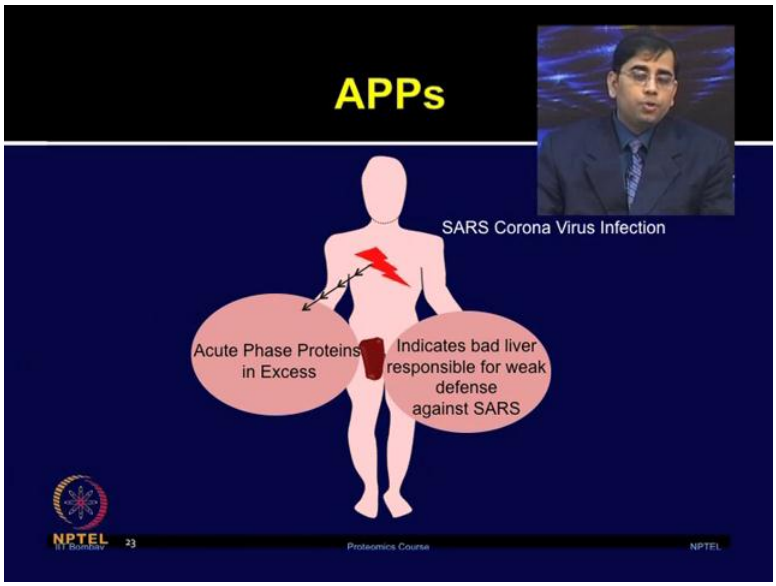

The active responses, and oxidation associated injuries may play a major role in the SARS pathogeneses. The acute phase proteins found in these SARS patients suggested, that the health status of liver in these patients may be affected there difference against the SARS virus infection. So, some of these information authors are able to obtain, because of the proteomic analysis of these plasma samples obtained from SARS patients. After discussing two case studies, one on the drug action of malaria parasite, two different drugs. and other the plasma proteome alteration due to the SARS infection. Now, proteomic investigations are quiet promising, but the major limitation of using the conventional 2 dimensional electrophoresis. If we remember earlier lectures, we discussed that why some of the inherent limitations of these 2DE need to be overcome with the some more refined techniques and that is where 2 DIGE came into the play.

So, the quantitative protein expression analysis that remains challenging in proteomics, 2 D electrophoresisis well suited to separate and analyze proteins, as well as their iso forms, and the optical detection of proteins with the florescent tap provides the excellent dynamic range. So, can we combine these 2powers, one of conventional 2 dimensional electrophoresis of separating thousands of protein, and other sensitive optical florescent dyes which could be highly sensitive.

So, in 1997 Unlu and collegues, they combine these two properties of 2 DE and fluorescent tap, and applied this method for DIGE quantitative protein analysis. So, let us

look at some case study on the 2 dimensional differences in gel electrophoresis, but before we touch upon the case studies, let me give you an overview of the DIGE technique.

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So, among 2DE and DIGE which of these two techniques, would be better to separate serum protein samples obtained from large number of patients in a clinical trial.

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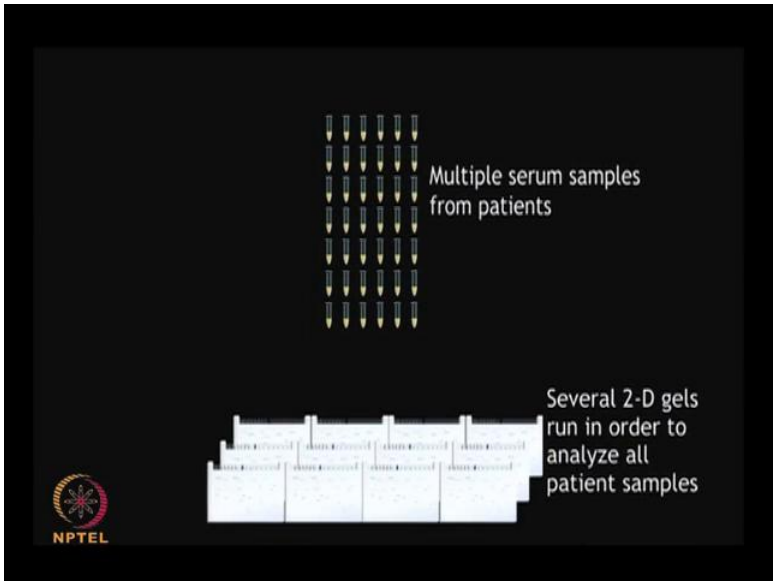

These are multiple serum samples from patients 2 dimensional electrophoresis, although a very useful technique, but it may not be the best option in this case to analyze serum proteins from large number of patients.

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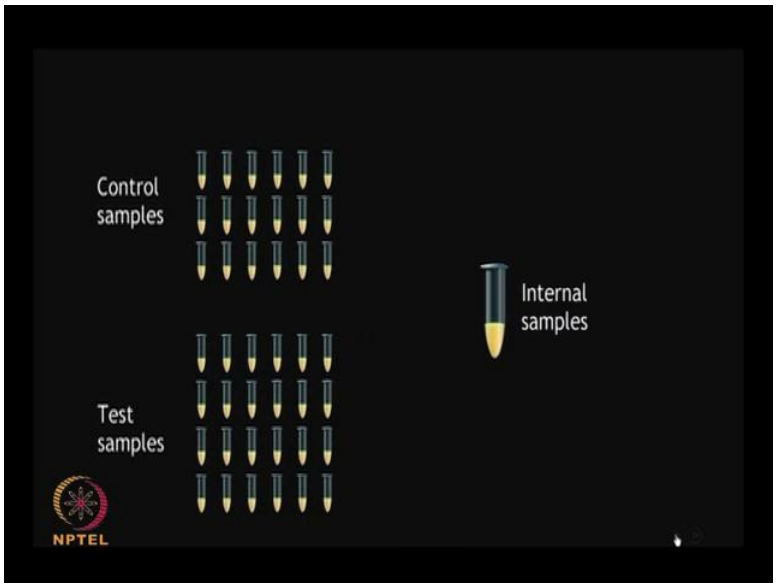

In this case, DIGE will be extremely valuable tool for analysis of large number of samples, simultaneously without having to overcome the problem of gel to gel variations.

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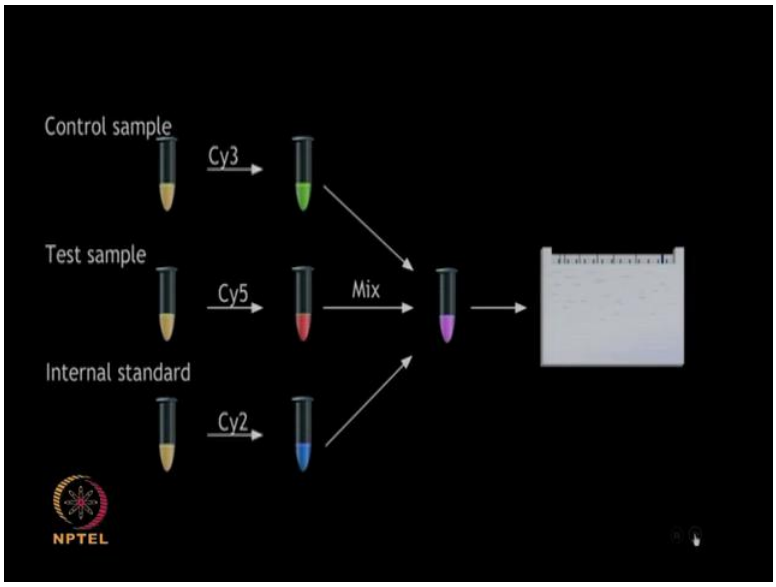

In DIGE gels the control, and test samples can be differentially labelled by using the cyanine dye DIGE, and then run on the single gel. The poured internal standard for DIGE is prepared by mixing equal amounts all the samples that need to be run on the gel, and this prevents the problem of gel to gel variation.

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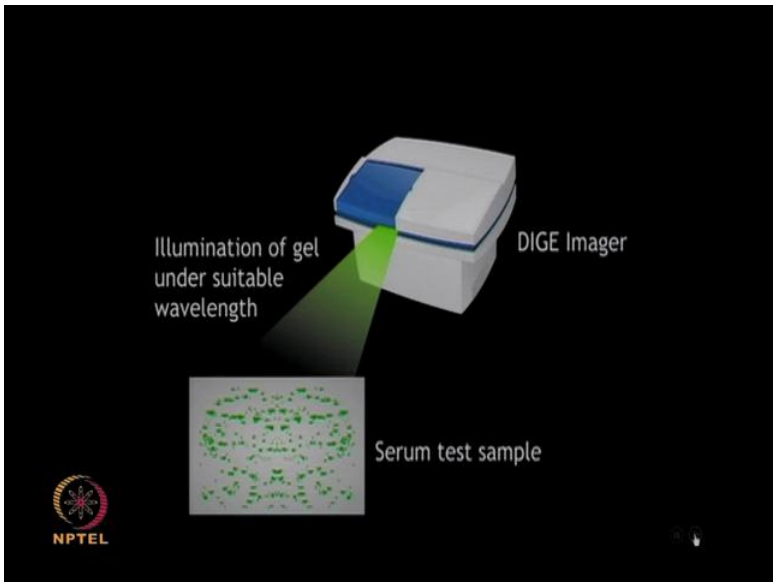

From the same gel 3 different images can be obtained for Cy2, Cy3 and Cy5.Therefore, there will be no reproducibility issue, and various artefacts can be eliminated further clinical or large number of sample analysis, which we have discussed earlier. So, I will just give you an overview, and for more detail protocols and detailed work flow please refer to the previous lectures. So, the main aim for the development of difference in gel electrophoresis was to overcome the inherently poor reproducibility of conventional 2 dimensional electrophoresis.

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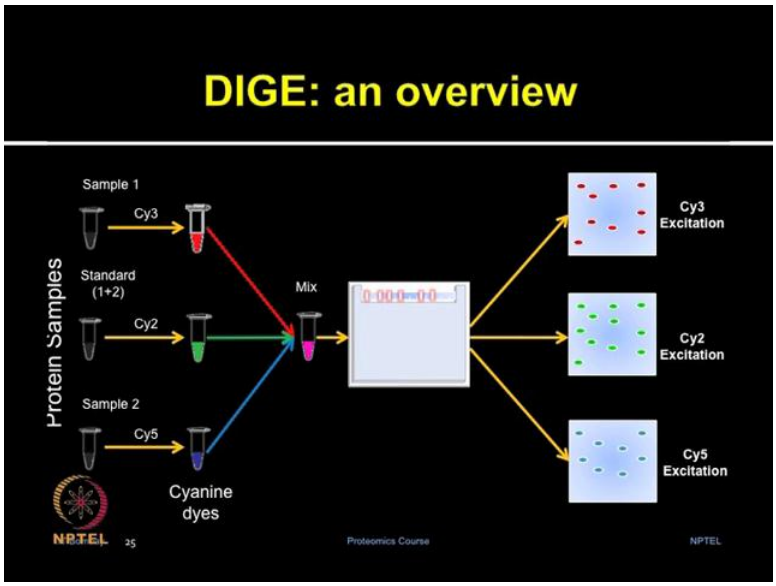

So, DIGE is quiet sensitive technique with less than one femtomolar of proteins which can be deducted, and it can enable the linear detection of very broad dynamic range of the proteins. So, as you can see in this slide the protein samples directly have control, and treatment those are labelled with two different dyes Cy3 and Cy5,but a small aliquot of both of these samples is mixed together to make a internal pool.

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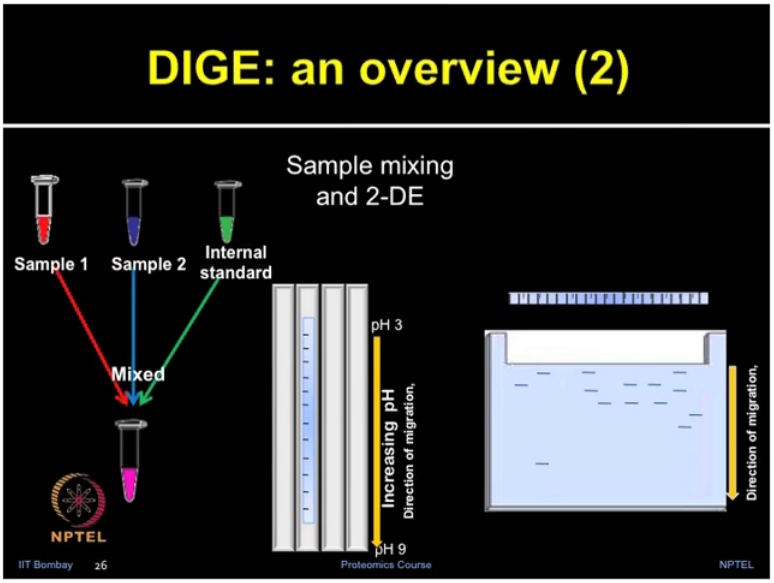

That internal pool is labelled with third dye Cy2.Now, all these three protein sample are mixed together in one tube, which contains both control treatment, as well as the

reference spots from the internal pool. All these protein mixture are separated in the first dimension on the same strip, and then the same gel can be scanned with the three different wavelengths to obtain the images for the Cy2, Cy3 and Cy5.So, in the conventional 2 dimensional electrophoresis the gel to gel variation, which comes from the acrylamide polymerization, electrical p H and thermal fluctuations in different gels that can be overcome in the DIGE gels, because all the protein separation is going to happen on the same gel.

So, all those artefacts can be minimized by using DIGE approach. So, in the slide it is shown that the three samples are mixed, and then isoelectric focusing is performed from the poor sample, and then this strip is placed on the SDS page gel for the protein separation in the second dimension. So, overall DIGE provides very uniform staining from gel to gel, and shows high sensitivity and linear dynamic range of detection for the expression profiling of complex biological samples. See, for aim is to resolve thousands of proteins, and cover comprehensive proteome coverage, then DIGE is a very good platform.

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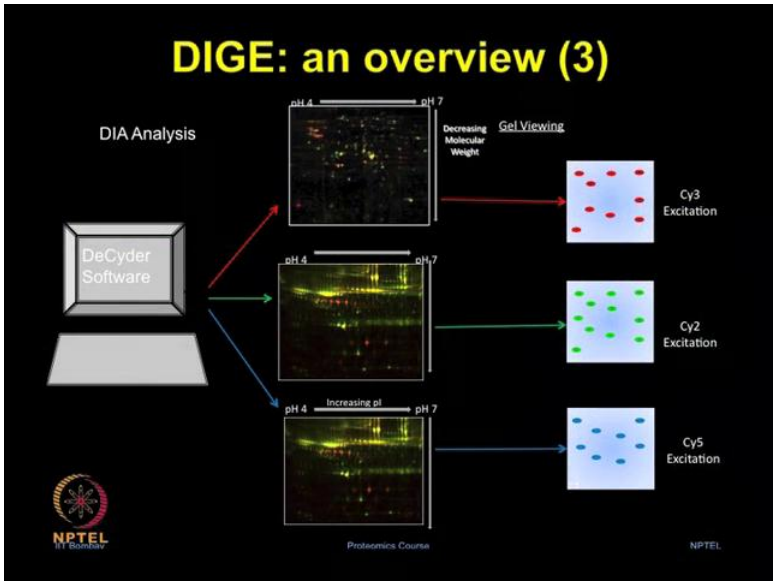

Especially, if you want to do the comparative or differential proteomic analysis, because you're gel to gel variations and other variations will be minimized, and DIGE will provide the very high sensitivity.

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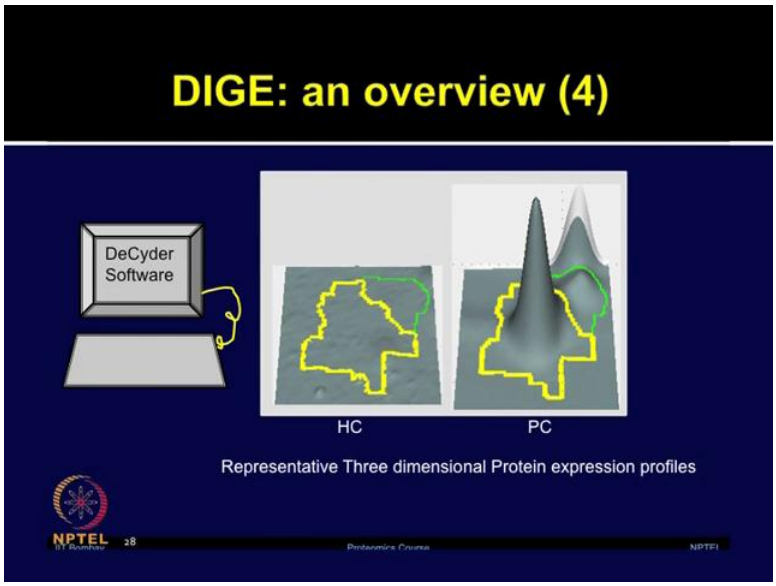

So, once you have run these gels, now from the same gel the images can be obtained, three images of your control and the treatment, and this can analyzed from different software, such as DeCyder software, and then by looking at three dimensional views and the statistical data, then these proteins can be considered as interesting for further investigations.

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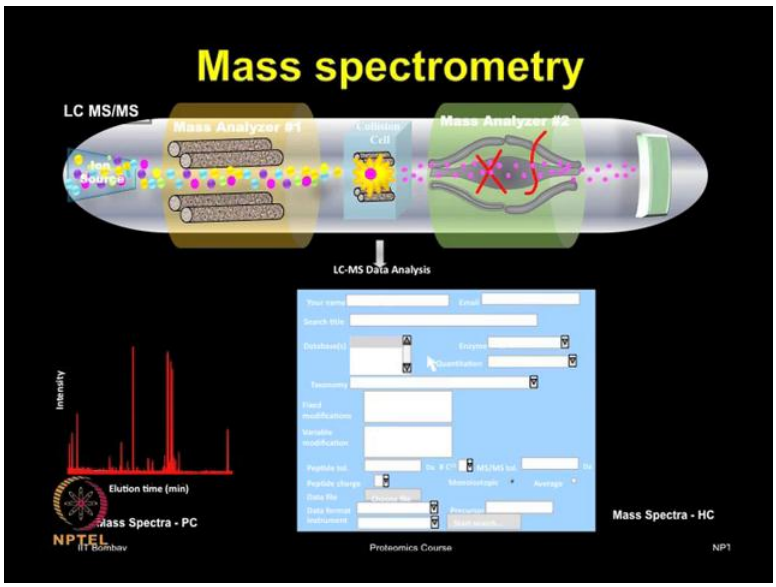

Once the spots are analyzed and excised from the gels, then the same tradition you have to follow, you can use any of the mass spectrometry platforms, and then obtain the m s

spectra for further analysis using different type of bioinformatic tools, such as mascot. So, overall the DIGE method is far more superior in terms of the reproducibility as compared to the conventional 2 dimensional electrophoresis, and for the quantitative accuracy. Therefore, applications of 2D DIGE can be found in virtually all major biological research areas. If you see the recent publication, you will appreciate there are several paper on each of the biological system for different type of applications, whether its cell signalling, it is looking at developmental biology, looking at plant proteomic analysis, neurosciences, clinical studies, different type of diseases including cancer, you will find there are hundreds of publications available, which have employed the power of 2D DIGE technique.

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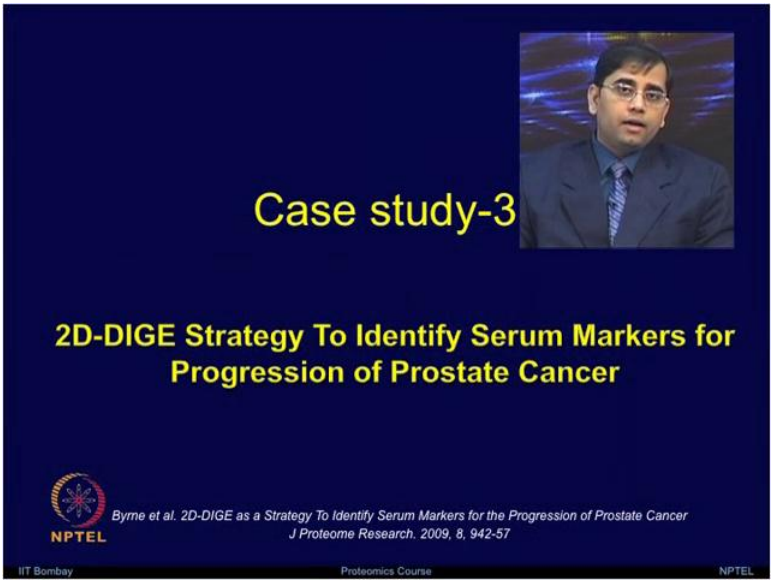

So, let us talk now a new case study, case study 3 on 2D-DIGE as a strategy to identify serum markers for the progression of prostate cancer. Study by Byme et al, published in 2009. So, this study authors aimed for identification of serum markers by depicting the progression of prostate cancer, by using difference in gel electrophoresis technique.

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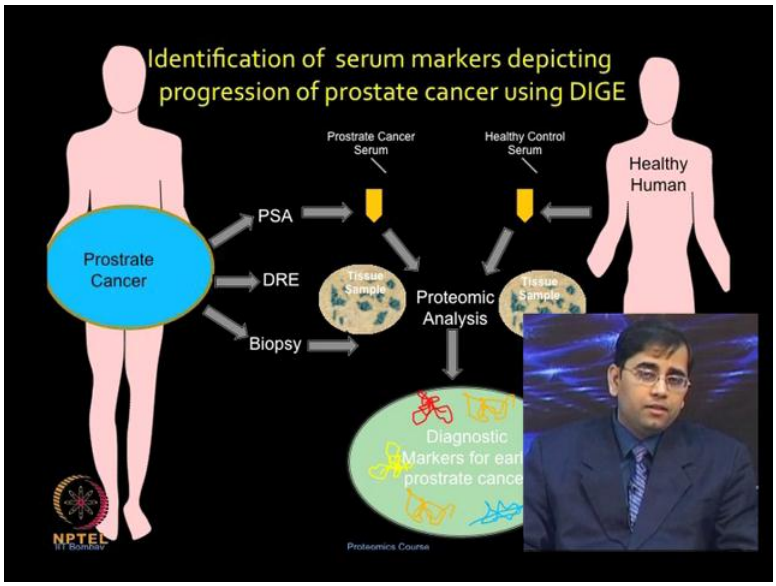

So, prostate cancer is recognized as a significant problem in older male population, the prostate cancer screening relyheavily upon testing for the higher level of prostate specific antigen, also known as PSA within the peripheral circulations. So,PSA is very sensitive marker, but the lot of discussion on reliability and specificity of PSA for the prostate cancer. Reason being that the level of PSA is also high in benign prostatic hyperplasia or prostatitis. So therefore, there is lot of discussion whether one should rely on only PSA for detection of the prostate cancer. So, this study aim to identify some new markers in the prostate cancer by studying the serum proteome analysis.

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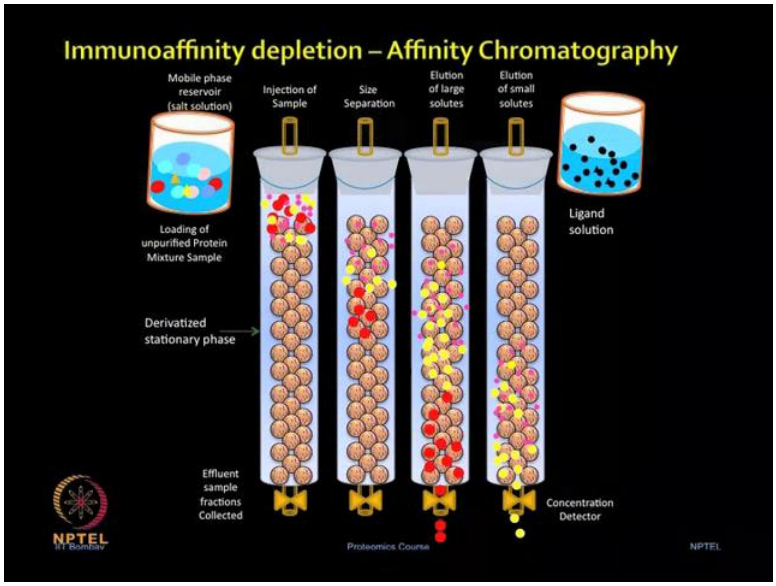

So, as you are aware, and in fact we have discussed the protein preparation from the serum earlier, so each of the biological sample poses lot of technical challenges, and serum is one among them where presence of highly abundant proteins, such as albumin and immunoglobulin, they result into the masking of low abundant proteins. So, to eliminate those high abundant proteins, authors used multiple affinity removal system from the Agilent technologies, and they removed most highly abundant proteins from the serum sample including albumin IGG antitrypsin IGA transferring and haptoglobins. So, after the abundant proteins were depleted from the serum sample, then authors move for the protein extraction and further analysis. So, the differential proteomic analysis was performed in the two different cohorts of histologically confirmed prostate cancer, with different grades of the disease.

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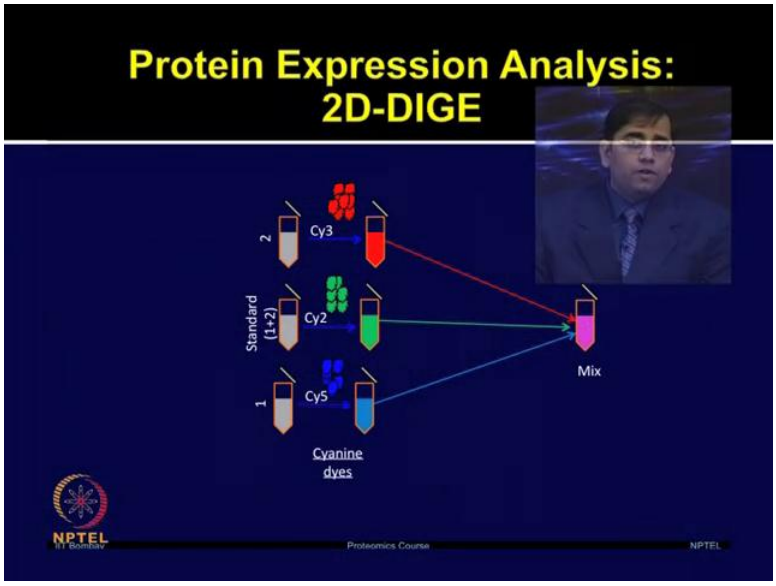

So, they used the patients with two different grading system based on Gleason grading, so the Gleason grading system that is used to help and evaluate the prognosis of men with the prostate cancer. So, depleted serum samples obtained from then patients with Gleason score 5 and Gleason score 7, were used for comparison and further analysis. As you can see in the slide these samples were first labelled with the Cy3,Cy5,and also the internal reference pools were made which were labelled with the Cy2 DIGE. These samples were then mixed, the depleted cancer serum form first cohort of Gleason score5,

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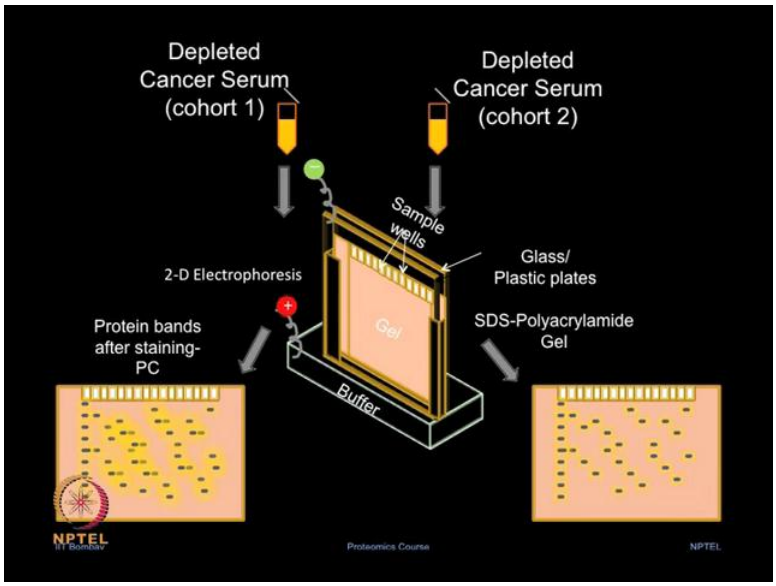

and second cohort of Gleason score7, those were mixed separated in the first dimension and followed by protein (( )) in the second dimension.

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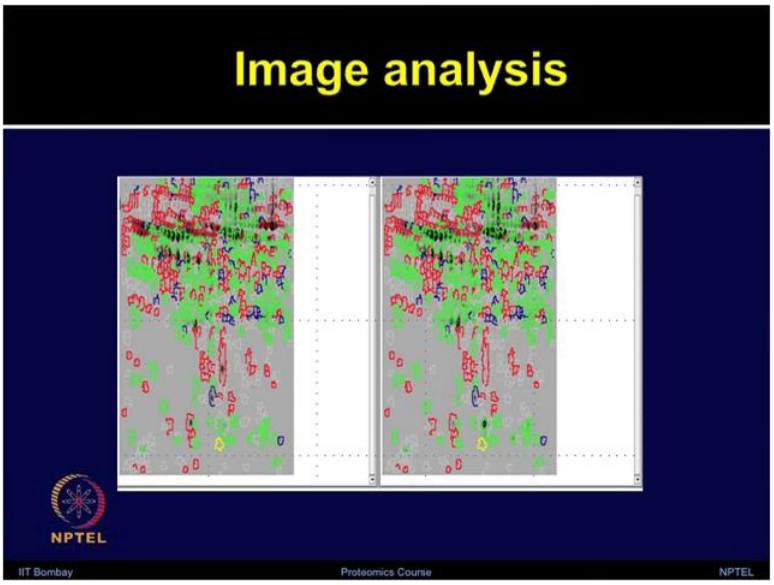

Now, when authors analyzed these DIGE images, they found at 63 protein spots were differentially expressed between the Gleason score 5 and 7 cohorts, and 13 of these proteins were statistically significant among these two populations. So, as you know analysis of these gels is always challenging, especially if you are looking at the conventional 2D gels, where you have separate gels obtained from each of this groups.

But, analysis in the DIGE gel is more automated, so if you remember our previous discussion in the DIGE gel analysis. This analysis is more automated and more straightforward, but still we have to through individual spots, and we have look for the how real, how significant those changes are, and you have to look at the 3D view the those spots to ensure that it is the reproducible among various control and treatment groups. So, the different level of analysis performed which we have talked earlier, but this just show the final output that 63 spots after all the analysis steps were considered significant.

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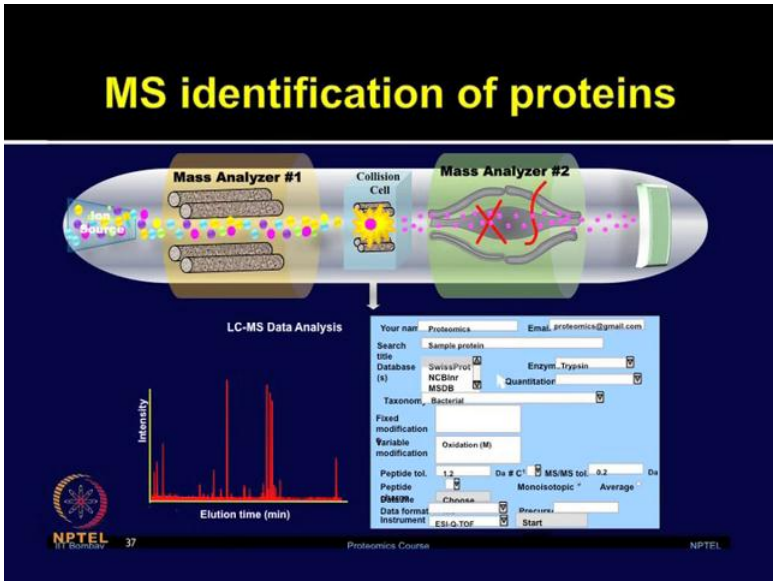

After2D-DIGE gel image analysis, authors excise those spots, and subjected for the mass spectrometry identification of proteins, so the proteins excised from the gels were analyzed by using finnegan l t q mass spectrometer, and data from these MS/MS experiments were analyzed by using bioworks browser by using sequest program.

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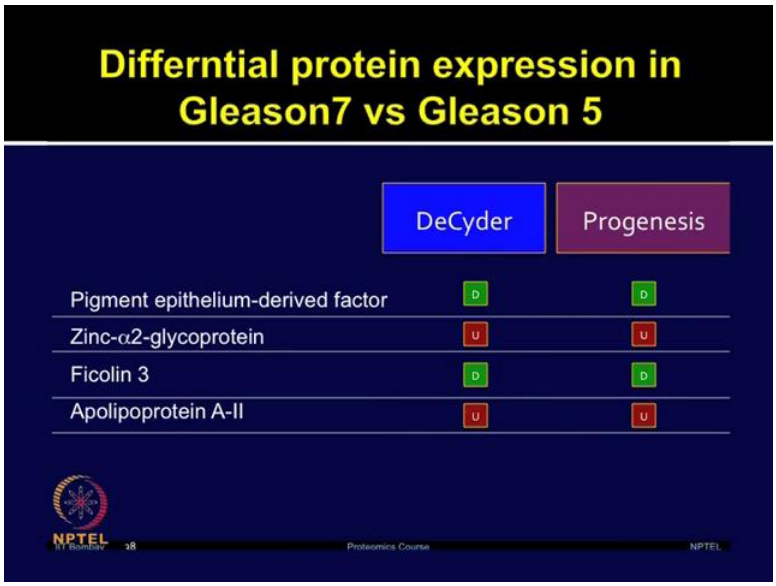

After mass spectrometry was done the identity of these proteins were established. Authors also tried analysis of these dye gels by using two different software packages, the DeCyder and Progenesis. Just so that, they are very confident that all the proteins which they are going to be analyzed for mass spec, those are very reproducible.

So, when the proteins which were common in both DeCyder and Progenesis, and the identity of those proteins were further established by using mass spec, those proteins included pigment epithelium derived factor, which was down regulated in both the cases, in DeCyder as well as Progenesis analysis. Zinc alpha 2 glycoprotein it was up regulated from the both the software analysis. Ficolin three was down regulated, and apolipo protein a 2 was upregulated in the both the software analysis.

So, these spots were having similar or uniform trend regardless of what software they analyzed, and then author thought these proteins could be interesting for further validation. So, these are only few proteins which I have shown here, there is a detailed list of proteins which one can study in the original manuscripts. For validation authors employed various techniques including western blots, enzyme linked immunosorbent assays or Elisa's, and also immunocytochemistry.

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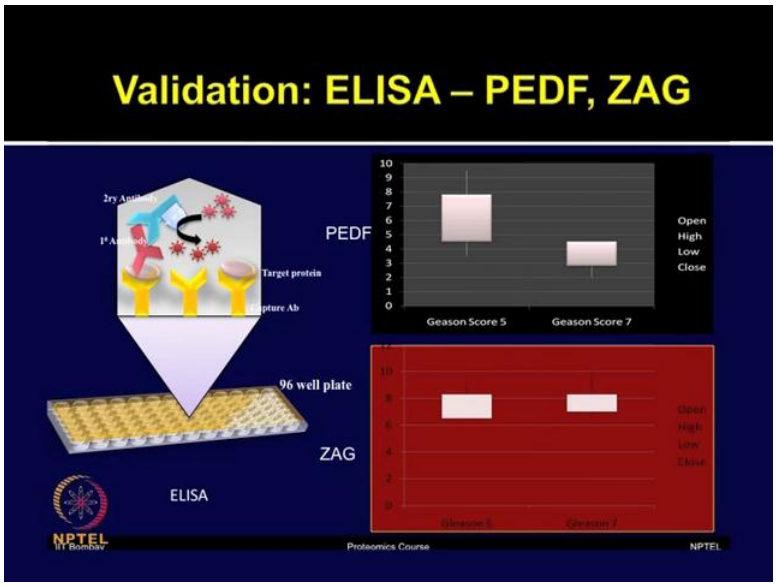

So, the pigment epithelium derived factor PDEF and zinc alpha 2 glycoprotein also known as ZAG those proteins were further validated by the ELISA technique. So, the PDEF levels were quantified by using ELISA kit, and results demonstrated as you can see in the slide that the statistically significant decrease in the PDEF in the Gleason score 7 depleted serum groups. Whereas, the results for zinc alpha 2 glycoprotein ELISA analysis, which is shown in the red in the bottom panel that indicated one point fourfold increase in the zinc alpha 2 glycoprotein absorbance in the Gleason score 7 group. So, these studies, this ELISA validation confirm their findings from the2D-DIGE experiments.

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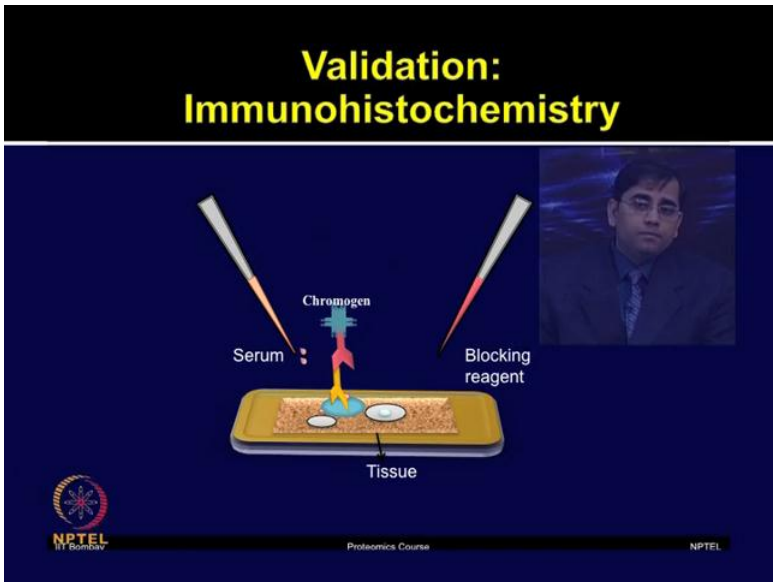

Authors also employed immunohitochemistry, or ICS for validating the pigment epithelium derived factor PDEF and zinc alpha 2 glycoprotein, so that they are very confident that the proteins which they have identified from the proteomic profiling those are real, and they also tested those on the independent tissue samples. So, from this paper the major conclusions were that serum markers, which are reflective of the pathological grade, and stage could be beneficial for the identification of appropriate treatment strategies.

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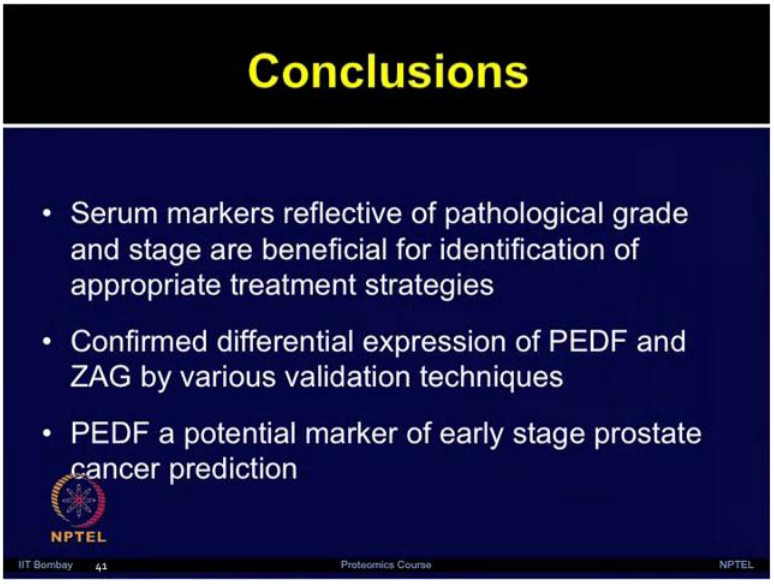

Authors confirmed that differential expression, PDEF and ZAG can be performed by using various techniques, such as western blot, ELISA and immune histochemistry. Based on the studies and the follow up experiments, they concluded that PDEF could be a potential marker of early stage prostate cancer prediction. However, more studies and follow up required on the large number of patients before it can be established a good bio marker So, in summary in today's lecture we finished two case studies, on 2 dimensional electrophoresis, which were continued from the previous lecture.

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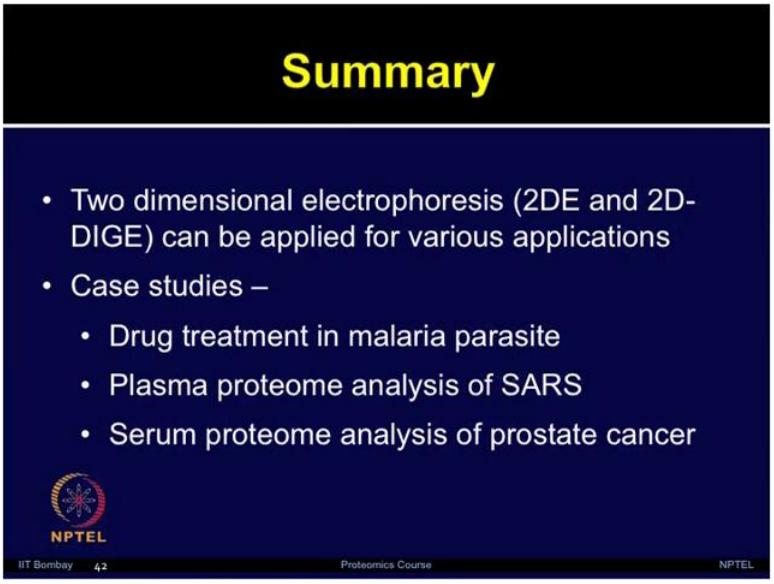

So, we discussed about different type of drug treatment in malaria parasite plasmodium falciparum, we also discussed about the SARS proteomealteration in the plasma due to the SARS virus infection, and after looking at these two conventional proteomic analysis by using 2DE, then we discussed the DIGE technique, the overview of 2D-DIGE was discussed, and then we talked about how we can use this technique for more applications, then as a representative example we took the study of prostate cancer, the serum proteome analysis.

So, one thing which you will observe that all the middle steps in the entire workflow of 2D and DIGE are very similar. It is a very first step in sample preparation, which is quite distinct in each of these two techniques, and it is quite unique for various type of biological samples. So, all your creativity and your modification those play major role during the very first step.

Once, you have obtained a good protein extract then same protein extract can be employed for various type of technologies, and then the workflow of all the steps which we talked earlier for 2D and DIGE, that remains very similar. So, in that regard you have not noticed too much difference in the overall applications, whether we have talked about plasma proteome analysis based on the conventional 2DE,or a serum proteome analysis of prostate cancer based on the DIGE technique, because at the end the you want to establish the aginity of the proteins by using mass spectrometry approaches. But how many proteins you can actually differentially express, you can establish? That fact is major discriminator between the these two techniques, you might lose lot of significant spots by using only conventional proteomic analysis, because you have apply large number of gel for those comparisons.

But, regardless of the pitfalls of each of these techniques, you may appreciate that there is lot of power of these techniques, and these can be employed for any biological application. You pick and application of your choice, and I am sure you will be able to answer those by employing 2D or2D-DIGE techniques. Thank you.

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