Introduction to Proteomics
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Lecture – 36
Proteomics Applications

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Welcome to the proteomics course. We started discussing about some case study two dimensional electrophoresis applications.

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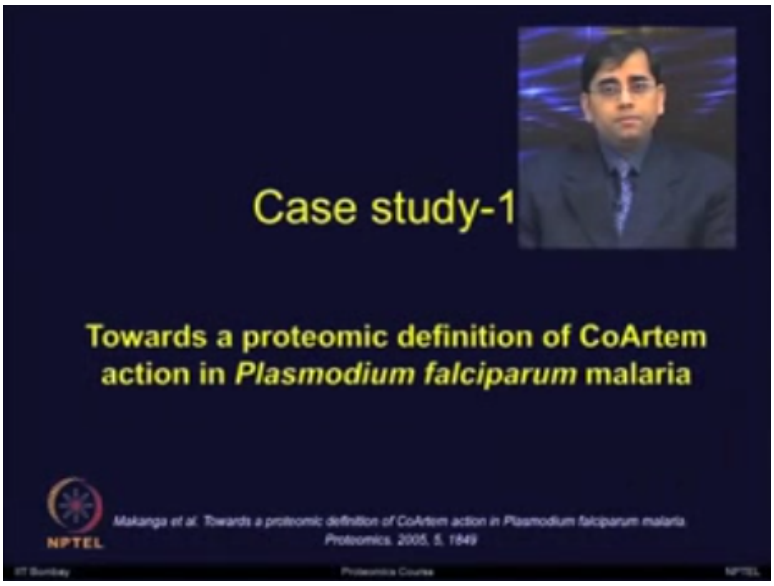

Let us start with the first case study, towards a proteomic definition of CoArtem action in Plasmodium falciparum malaria, a study by Makanga et. al. in 2005. So, as you know each year hundreds of millions of new malaria infection cases result in over 1 million deaths worldwide but due to the lack of effective vaccine and widespread resistance to the anti-malarial drug, still lot of deaths are happening and the malaria problem is still posing challenges for its control.

The anti-malarial therapy of chloroquine and primaquine, these have not been able to control the mortality rate because of the anti-malarial drug resistance development. So, therefore there is an urgent need for identifying new drug targets as well as understanding the course of action of these drugs by applying various type of high throughput techniques.

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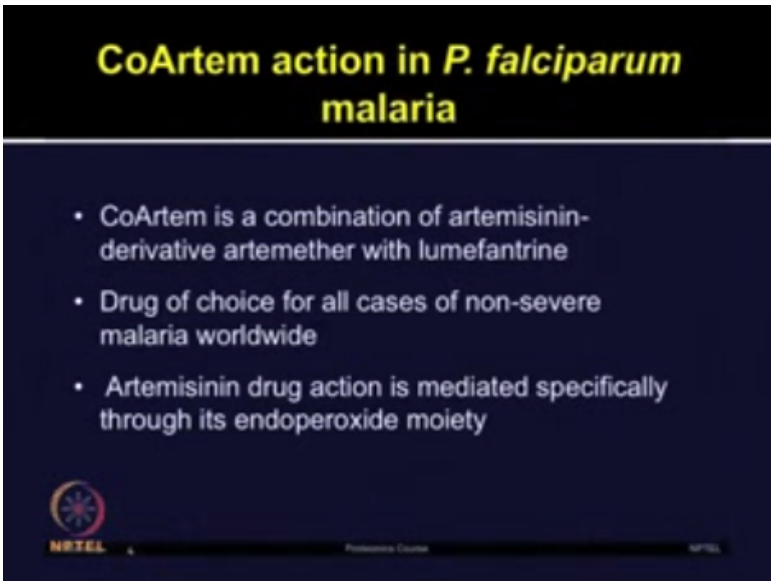

So, in this paper authors have discussed how two different drugs which are effective for the anti-malarial can be studied for looking at the proteome changes in the plasmodium falciparum parasite. So, CoArtem is a combination of artemisinin-derived artemether with lumefantrine. How, these two drugs behave and how the proteome changes occur due to the action of these two drugs were studied in this paper. (()) (02:31) applied proteomic approaches, the two-dimensional electrophoresis to study the proteomic alternation of each of these drugs.

So, these drugs are applied as a drug of choice for all cases of non-severe malaria worldwide. The artemisinin drug action is mediated specifically through its endoperoxidase moiety. However, the more detailed mechanism of action of these drugs are still unknown. So, the purpose of this study was to investigate the action of two active components of new anti-malarial CoArtem, artemether and lumefantrine on human malaria parasite plasmodium falciparum and author tried to look for the alterations in parasite proteome which were induced by each of these drugs.

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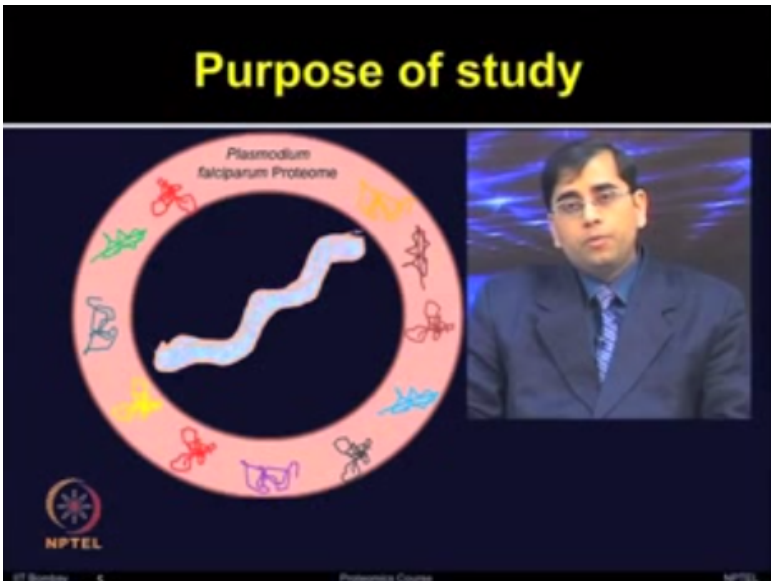

To obtain insight of the proteomic alteration, they separated the proteins on the two-dimensional electrophoresis gels and compared the response of the proteomic alteration based on these two drugs and then they identified certain proteins which were either commonly expressed due to these drugs or they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

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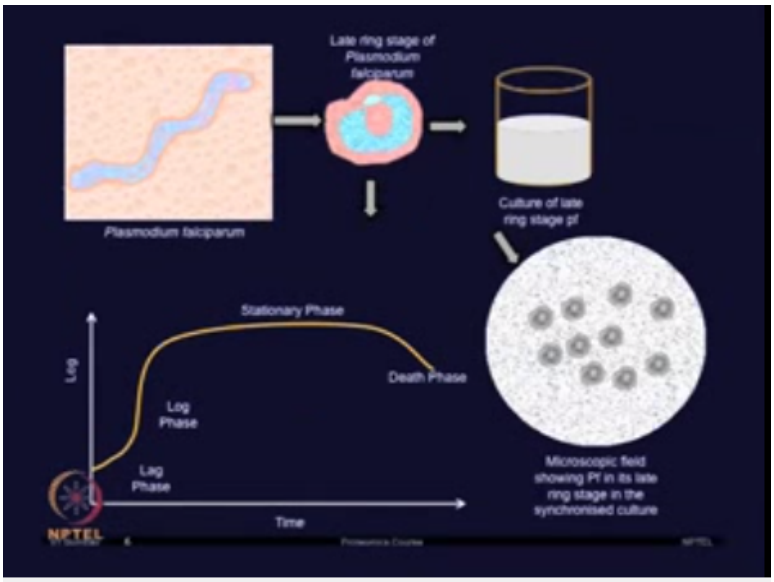

But before looking at the proteomic alterations, author first determined IC10, 20, 50 and IC 90 values for both the drugs, ARN and LUM. We will use the abbreviations now for artemisinin and lumefantrine. An effect of these concentration of drugs on parasite growth over 24 hours was

characterized. As you can see the growth curve in the slide. So, synchronised ring stage parasite cultures were harvested over 24-hour period after the exposure to the ARM and LUM. The parasite growth was determined by using hypoxanthine uptake assay.

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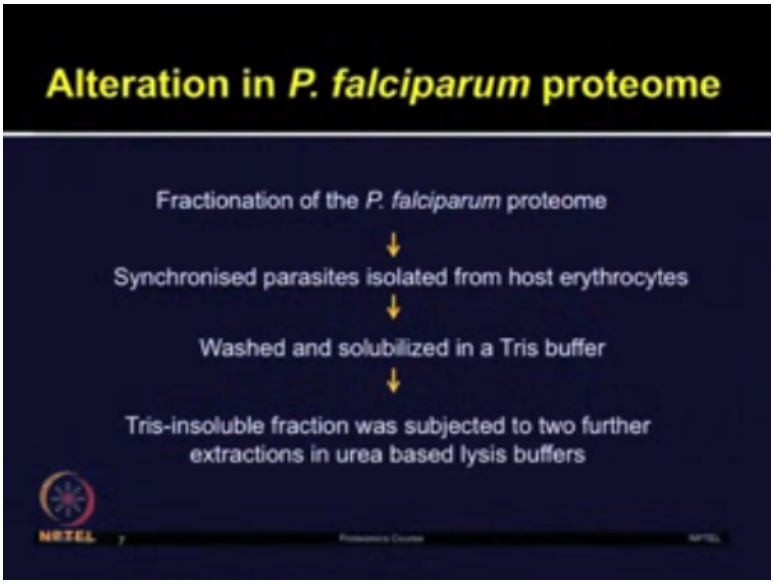

After establishing the culture conditions and the drug concentration, then author look for the proteomic alterations. So, first of all, they did fractionation of the P. falciparum proteome, synchronised parasites which were isolated from the host erythrocytes, washed those initially and then solubilised that in a Tris buffer recipe. The Tris-insoluble fraction was further subjected to extraction in the urea-based lysis buffer.

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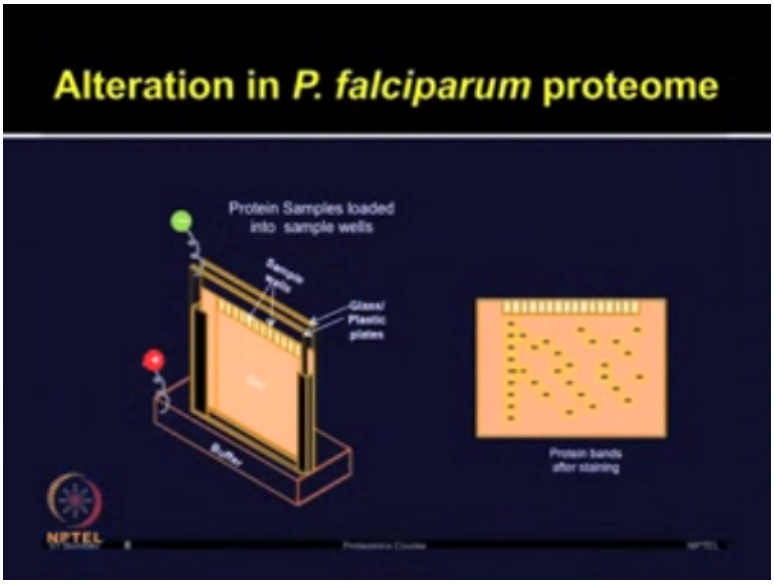

Once protein section was done, then authors used IPG strip off PH3 to 10 range for the first dimension separation of protein in the linear IPG strips. After IEF was done, they equilibrated these IPG strips and then applied that on 12.5% of the vertical SDL gel. After the second dimension separation based on the molecular weight, then these just were stained with the silver or Coomassie brilliant blue stains.

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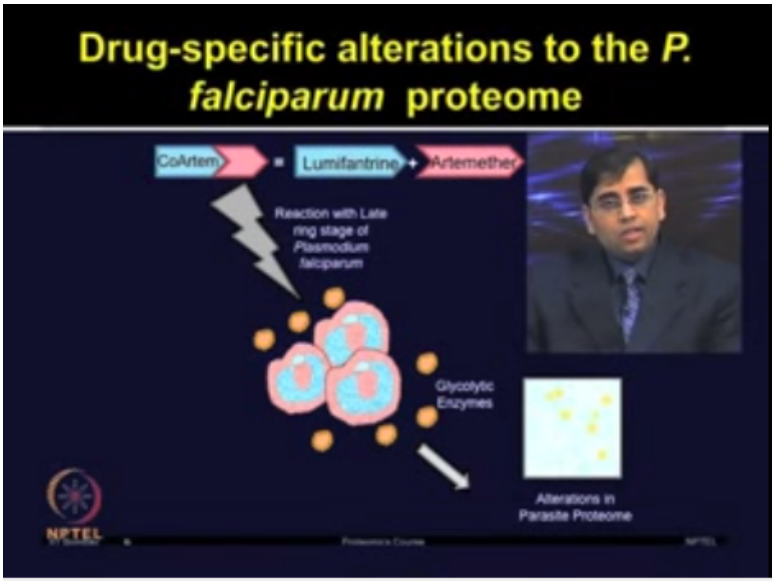

So, by employing two-dimensional electrophoresis and comparing the gel images by using the PD-Quest Software, authors are able to see that there is a differential proteomic response which is drug specific. So, quantitative analysis of alternate protein expression levels following exposure to the ARM and LUM were analysed and then those protein spots which were differentially expressed and it showed a significant wear further subjected to the mass spectrometry analysis.

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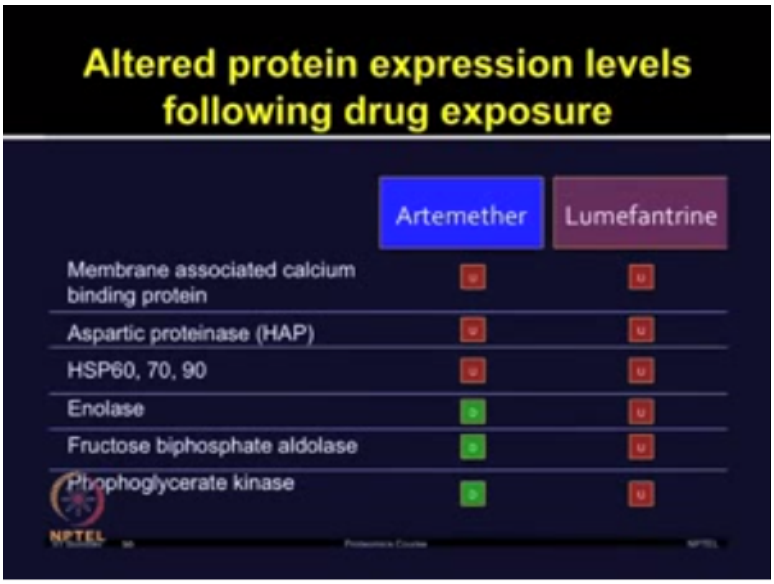

So, the comparative analysis of 2-D gels from untreated and the drug-treated parasite protein fractions provided direct and distinct alterations in parasite proteome following artemisinin or lumefantrine drugs. Certain proteins were identified. Few of those showed common response due to both drugs; however, there are certain proteins which showed opposite trends due to each of these drugs. Protein such as membrane, associated calcium binding protein was up regulated in both the drugs.

Aspartic proteinase was 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. There are certain protein such as enolase, fructose bisphosphate aldolase, and phosphoglycerate kinase. These proteins were down regulated in artemether treatment and up regulated in the lumefantrine treatment.

So, interestingly, the ARM treatment regulated in more than three fold down regulation of the glycolytic enzymes such as enolase, phosphoglycerate kinase, fructose bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase.

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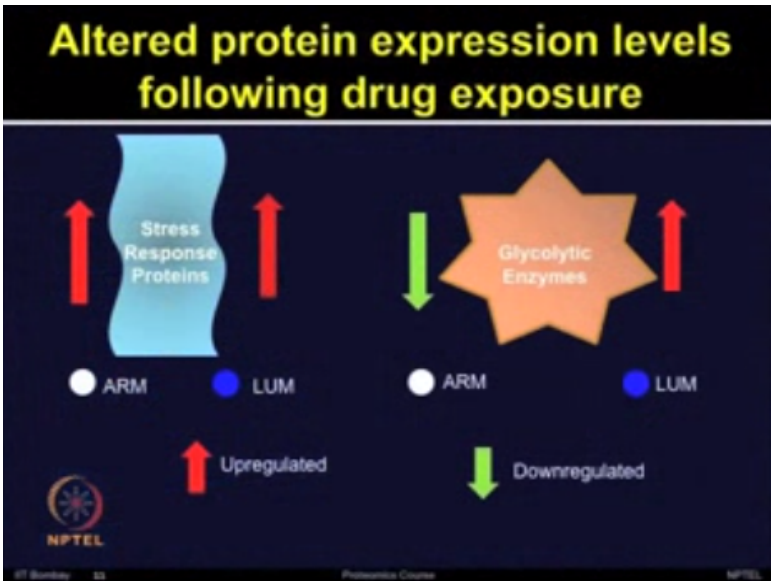

The expression of the same enzymes were also up regulated more than threefold due to the lumefantrine treatment; however, there are certain proteins such as the stress responsive proteins, like heat shock proteins which were commonly induced due to either of these drug treatments which looks like a general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

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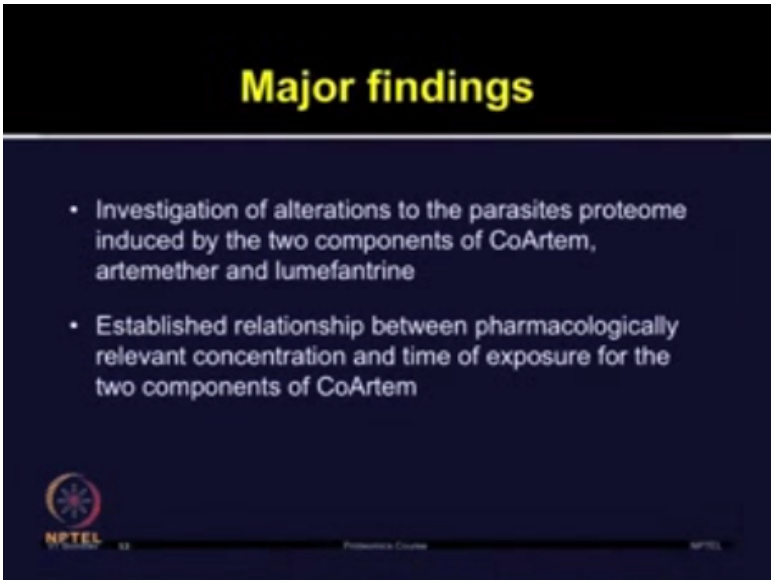

So, from this study the major findings were that authors successfully investigated alterations of parasite proteome induced by two components of CoArtem, artemether and lumefantrine. By using proteomic approach, they investigated specific and non-specific effects of two anti-malarial drugs in pharmacological relevant conditions. Expression of certain proteins were quite

interesting including a membrane-bound calcium binding protein which was up regulated 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. So, in this study authors aim for identification of serum markers by depicting the progression of prostate cancer by using difference in the electrophoresis technique.

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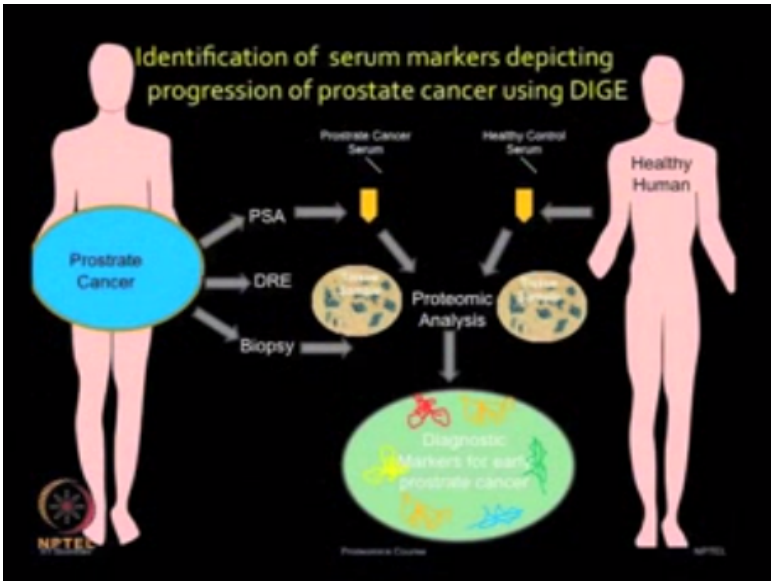

So, prostate cancer is recognized as a significant problem in older male population. The prostate cancer screening rely heavily upon testing for the higher level of prostate specific antigen, also known as PSA within the peripheral circulations. So, PSA is very sensitive marker but there are a lot of discussion on reliability and the 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 a lot of discussion whether one should rely on only PSA for detection of the prostate cancer. So, this study aims to identify some new markers in the prostate cancer by studying the serum proteome analysis.

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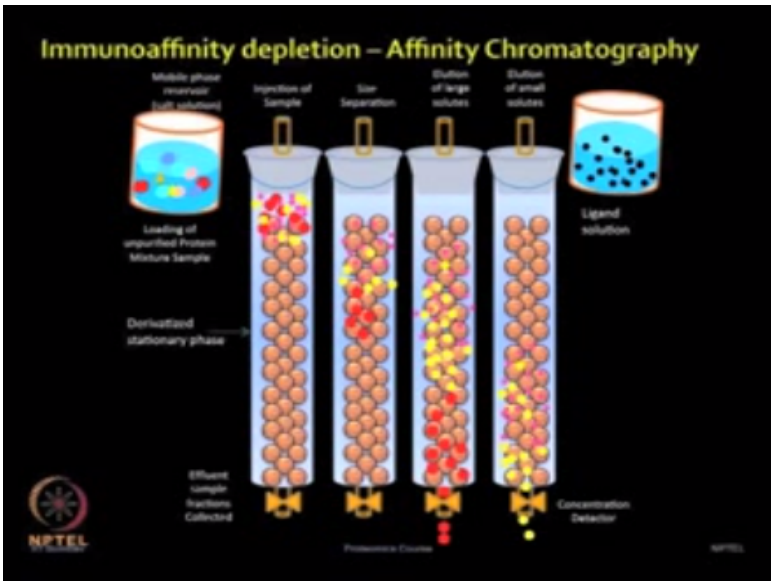

So as you are aware and in fact, we have discussed the protein preparation from the serum earlier. So, each of the sample poses a lot of technical challenges and serum is one among them where presence of highly abundant proteins such as albumin and immunoglobulin, they result into the masking of low 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 serum sample including albumin, IgG, antitrypsin, IgA, transferrin and heptoglobins. After the abundant proteins were depleted for the serum sample, then authors move for the protein extraction and further analysis.

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So, the differential proteomic analysis was performed in two different cohorts of histologically confirmed prostate cancer with different rates of the disease.

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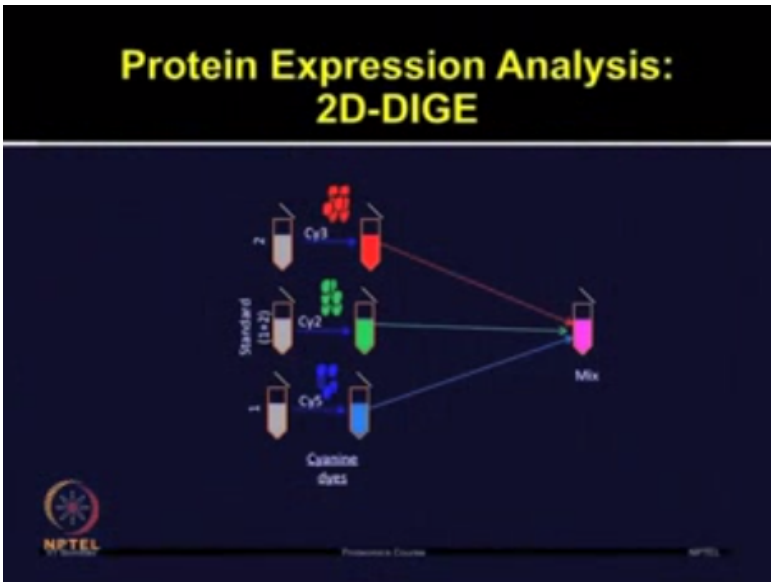

So, they used the patients with two different grading system based on the Gleason grading. So, the Gleason grading system that is used to help and evaluate the prognosis of men with prostate cancer. So, depleted serum samples obtained from the patients with Gleason score 5 and Gleason score 7 were used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

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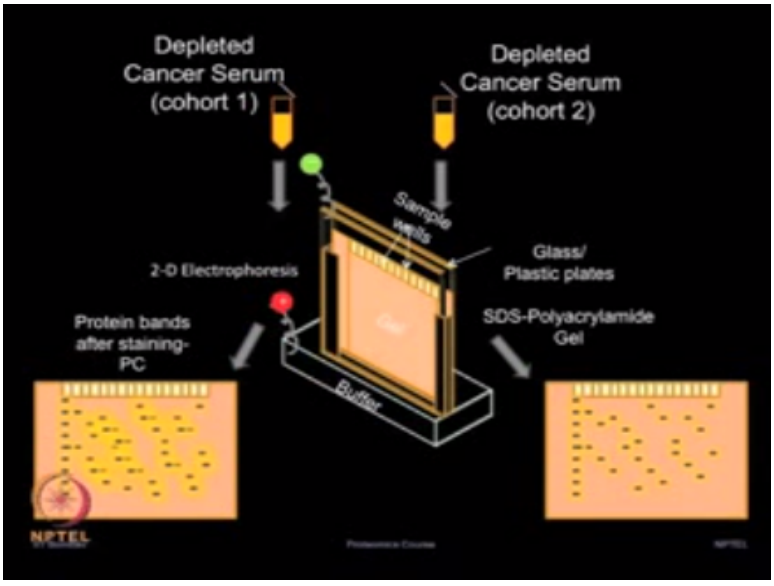

The depleted cancer serum from first cohort of Gleason score 5 and second cohort of Gleason score 7, those were mixed, separated in the first dimension and followed by proteins that separated in the second dimension.

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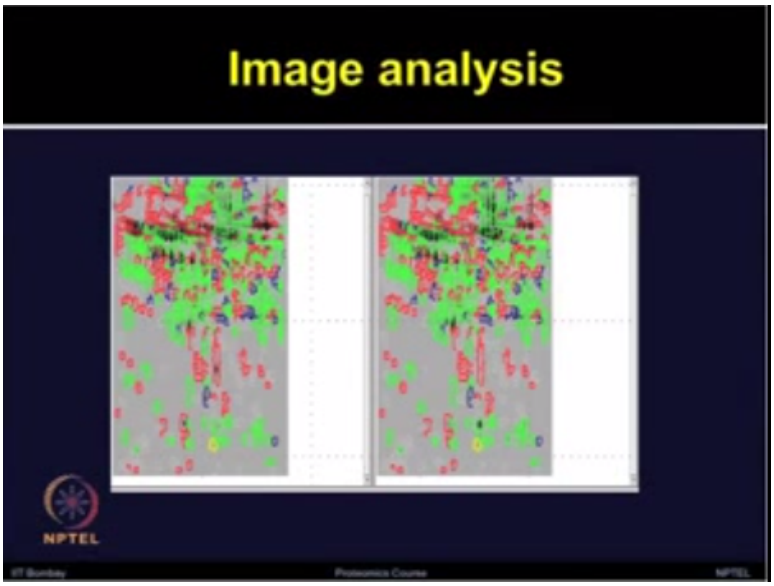

Now, when authors analysed these diced images, they found that 63 protein spots were differentially expressed between the Gleason score 5 and 7 cohorts and 13 of these proteins were statistically significant among these two populations. So, as you know analysis of the gels is always challenging, especially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

But analysis in dye gel is more automated. If you remember our previous discussion in the dye gel analysis. This analysis is more automated and more straightforward but still you have to go through individual spots and you have to look for how significant those changes are and you have to look at the 3-D views of those spots to ensure that it is reproducible among various control and treatment groups.

There is different level of analysis performed which we have talked earlier but this just shows you the final output that 63 spots after all the analysis steps were considered significant.

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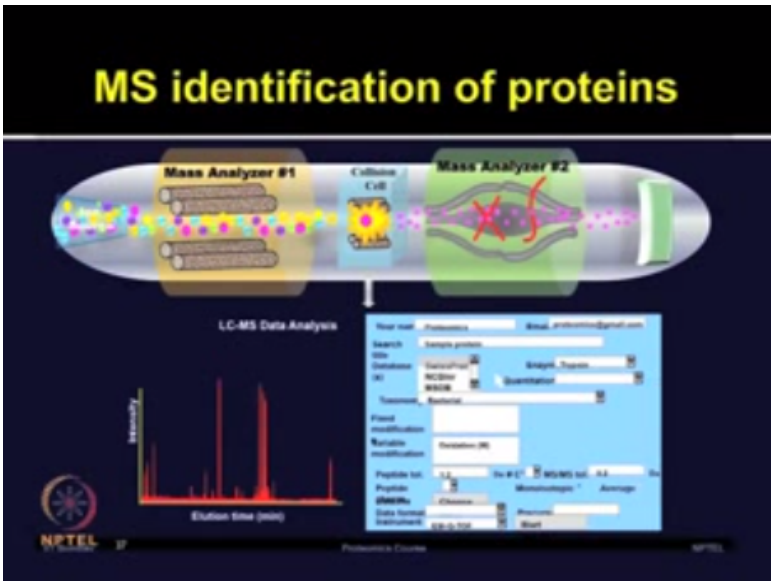

After 2-D dye gel image analysis, authors (()) (16:06) those spot and subjected for the mass spectrometry identification of proteins, so the proteins excised from the gels were analysed by using Finnigan LTQ mass spectrometer and data from these MS/MS experiments were analysed by using BioWorks browser by using C-Quest program.

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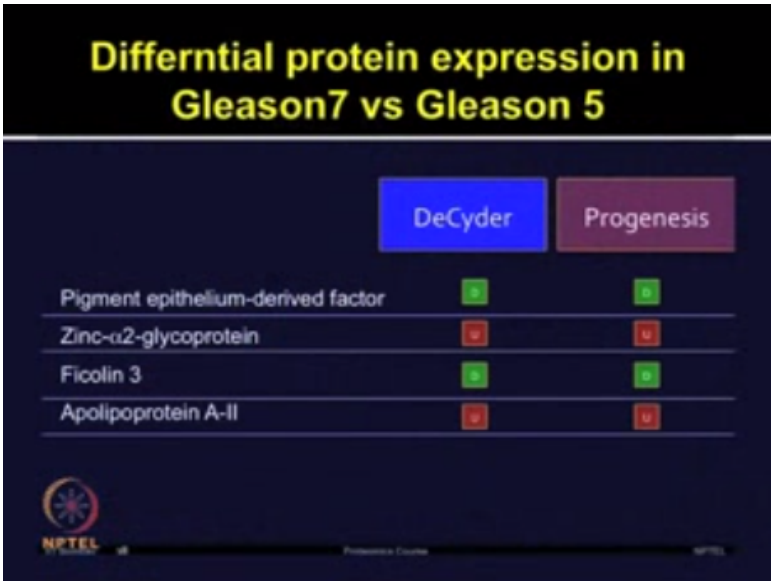

After mass spectrometry was done, the identity of these proteins were established. Authors also tried analysis of these dye gels by using two different software packages, the DeCyder and Progenesis just so that they are very confident that all the proteins which they are going to be analysed for mass spec those are very reproducible. So, among the proteins which are 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 related from both the software analysis. Ficolin 3 was down regulated and apolipoprotein A-II was up regulated in both software analysis. So, these spots were having similar or uniform trend regardless of what software they analysed and then author 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 the proteins which one can study in the original manuscripts.

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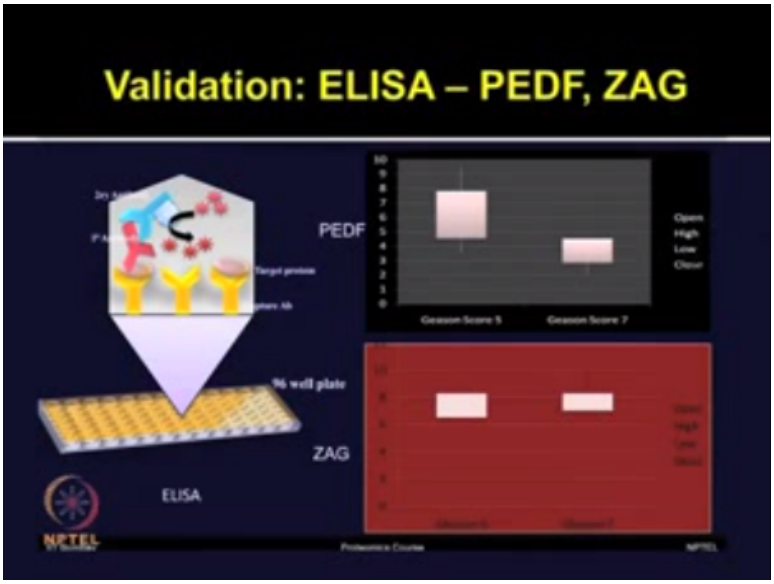

For validation, authors employed various techniques including Western blots, enzyme-linked immunosorbent assays or ELISA and also immunohistochemistry. So, the pigment epithelium derive factor PEDF and zinc alpha-2 glycoprotein also known as ZAG, those proteins were further validated by the ELISA technique. So, the PEDF levels were quantified by using ELISA kit and results demonstrative as you can see in the slide that the statistically significant decrease in the PEDF in the Gleason score 7 depleted serum group.

Whereas the results for zinc alpha-2 glycoprotein ELISA analysis which is shown in red in the

bottom panel that indicated a 1.4-fold increase in the zinc alpha-2 glycoprotein absorbents in the Gleason score 7 group. So, these studies, this ELISA validation confirm their findings from the 2-D dye experiments.

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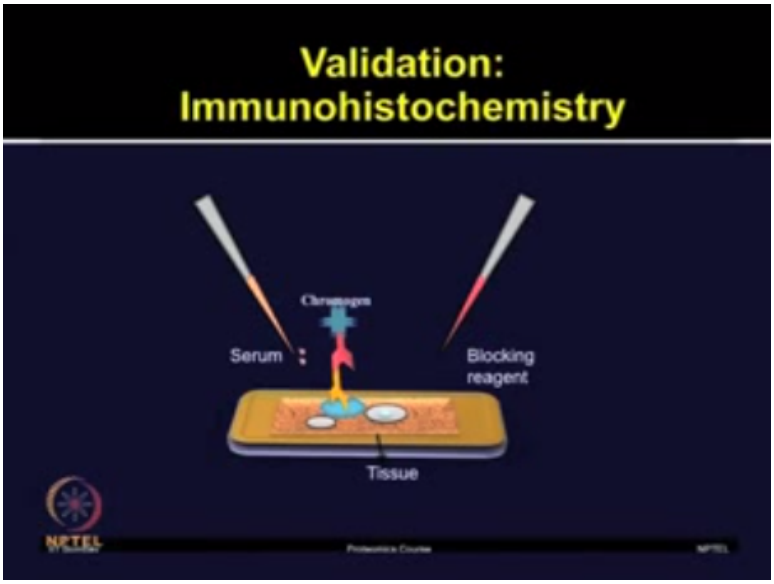

Authors also employed immunohistochemistry or IHC for validating the pigment epithelium derived factor PEDF and zinc alpha-2 glycoprotein. So, that they are very confident that the proteins which they have identified from the proteomic profiling, those are real and has also tested those on the independent tissue samples.

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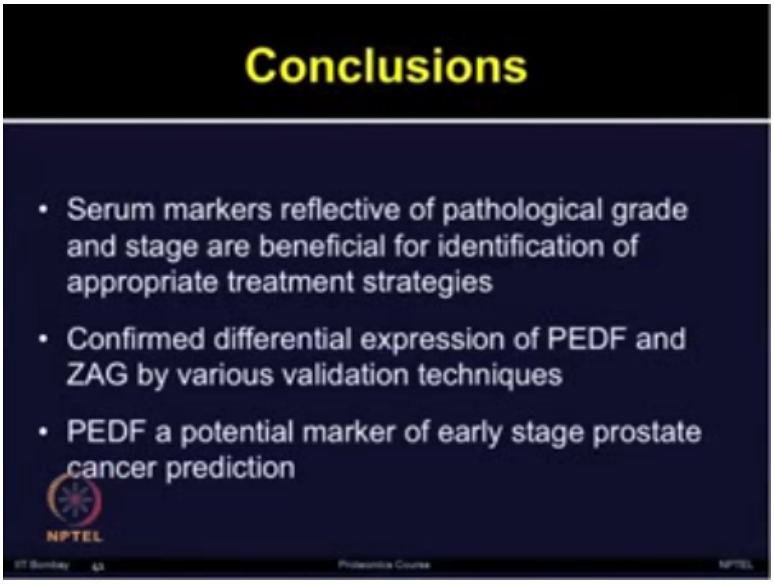

So, from this paper the major conclusions were that there are markers which are reflective of the

pathological grade and stage could be beneficial for the identification of appropriate treatment strategies. Authors confirm that differential expression of PEDF and ZAG can be performed by using various techniques such as Western blot, ELISA and immunohistochemistry.

Based on their studies and the follow-up experiment, they concluded that PEDF 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 establish as a good biomarker.

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Let us now discuss few applications of SILAC briefly. The SILAC method is very promising for any cell line.

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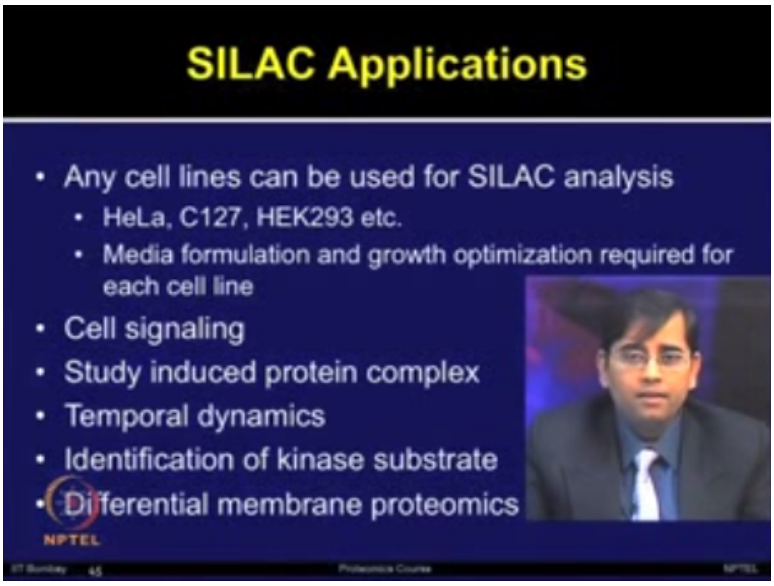

So, this method can be applied for initial lines, whether it is HeLa cell, C127, HEK293 or different type of cell lines people have shown. However, the media formulation and the growth optimisation is required individually for each cell line. SILAC applications have been demonstrated in different applications such as cell signaling, studying the induced protein complexes, studying temporal dynamics, identification of kinase substrates, studying differential membrane proteomics.

So, there are various applications, we will have a look on some applications now.

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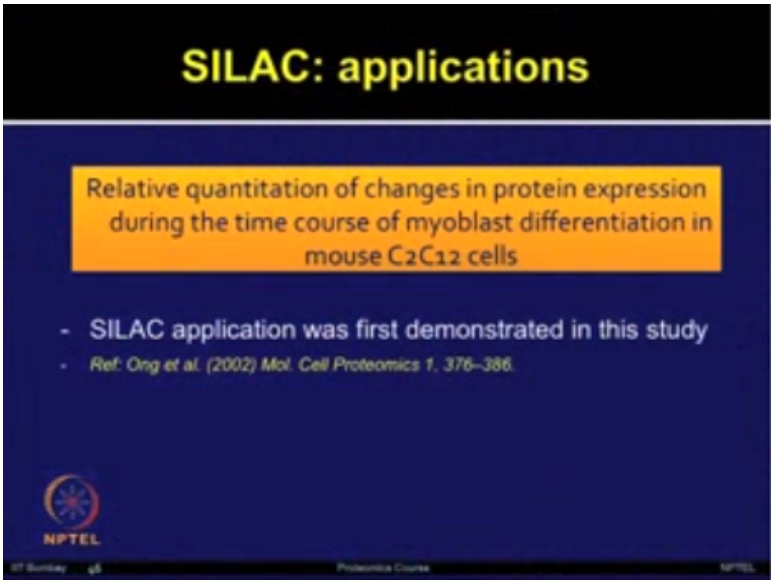

So Ong et. al. in 2002 published a paper in molecular cell proteomics which was the first SILAC

application demonstrated where they used relative quantitation of changes in protein expression during the time course of myoblast differentiation in mouse cells.

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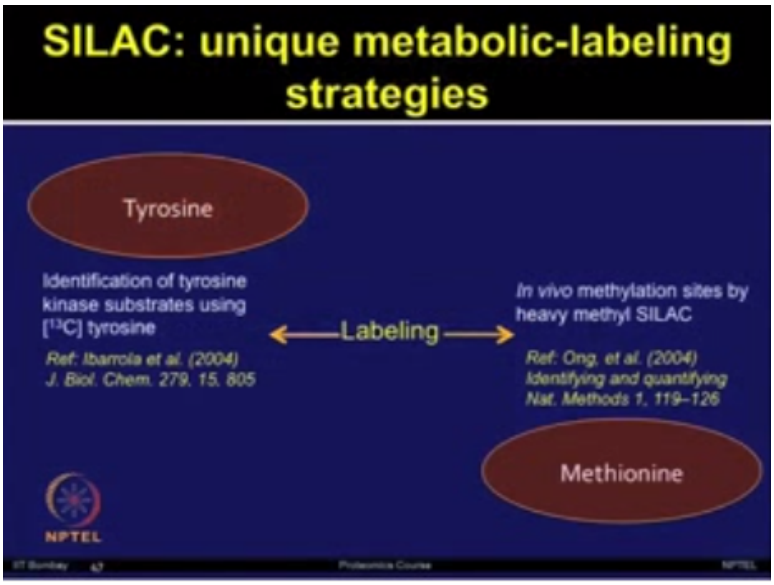

Researchers have reported various unique metabolic labelling strategies. For example, by using tyrosine, the identification of tyrosine kinase substrates using 13 C-tyrosine, labeling is also performed by using methionine, the in vivo methylation sites by methyl SILAC.

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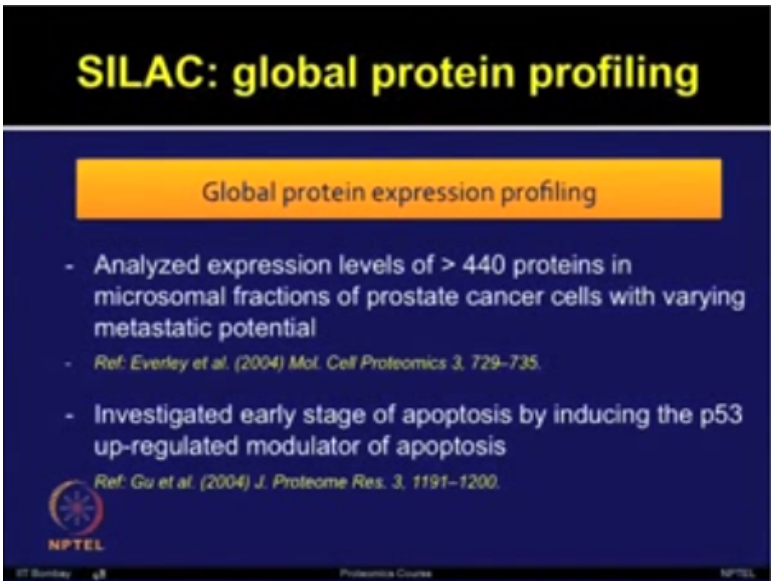

There are numerous studies based on the global protein expression profiling using SILAC method. I am just highlighting some of the very earlier studies which set a path for performing these proteins expression profiling. So, study by Everly et. al. in 2004 analysed the expression

level of more than 440 proteins. In the microsomal fractions of prostate cancer cells with very metastatic potential. Another study by Gu et. al. investigated the early stage of apoptosis by inducing the p53 up regulated modulator of apoptosis.

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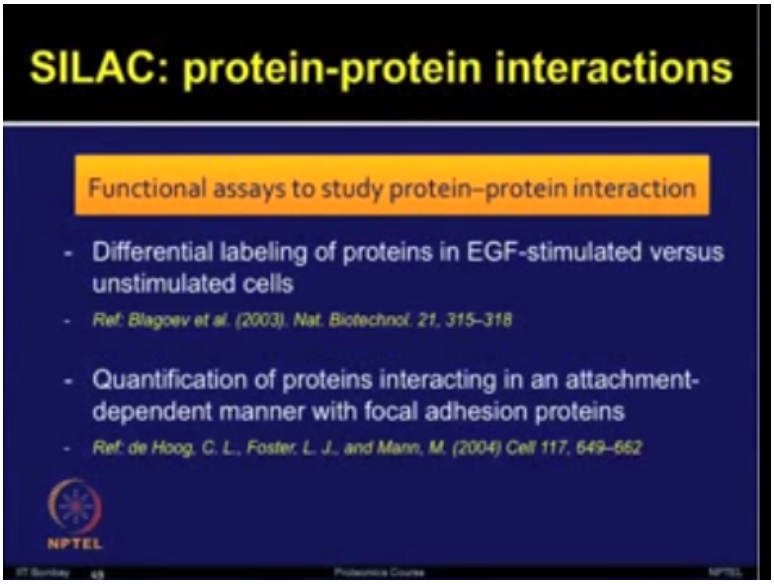

SILAC has also been used for functional assays to study the protein protein interactions. A study by Blagoev et. al. used the differential labelling of proteins in EGF is stimulated versus unstimulated cells, a study by de Hoog et. al. with quantification of proteins interacting in an attachment-dependent manner with focal adhesion proteins. These are just few example of studying the functional assays and performing protein interactions using SILAC.

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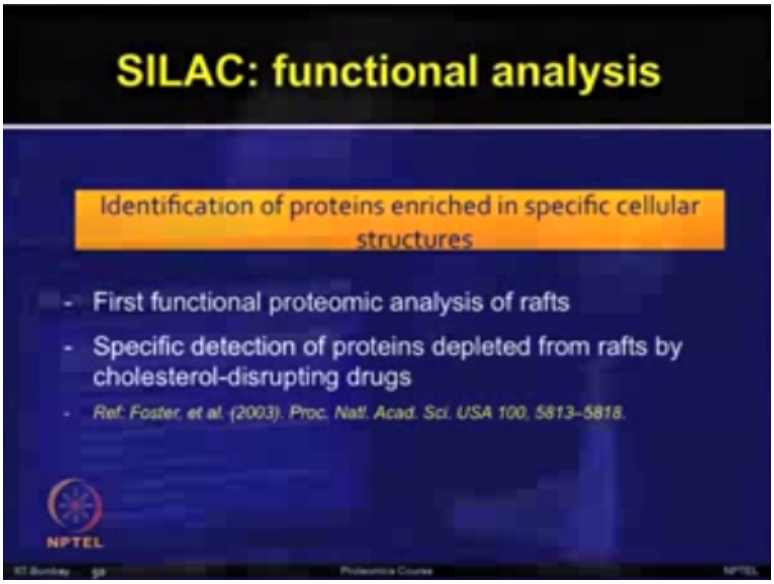

The identification of proteins which are enriched in specific cellular structures, a study by Foster et. al. used the first functional proteome analysis of rafts and they showed the specific detection of proteins depleted from the rafts by cholesterol disrupting drugs.

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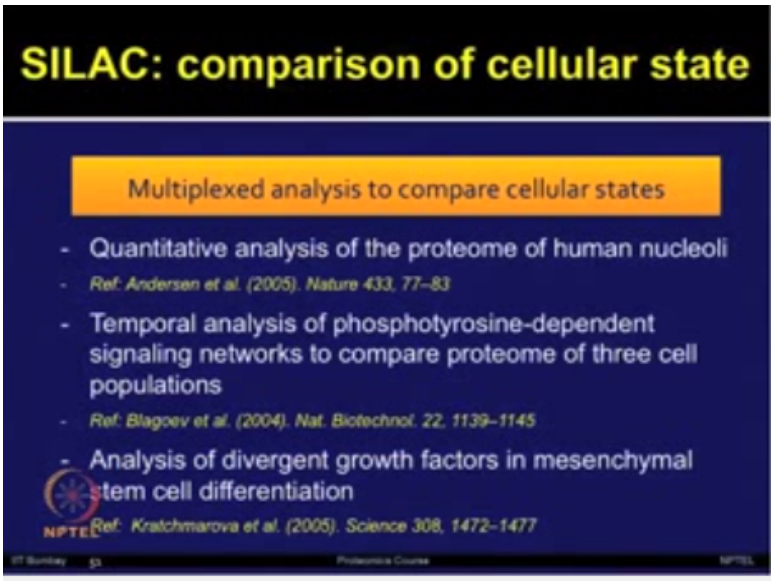

SILAC has been widely used for multiplexed analysis to compare the cellular states. Anderson et. al. shown the quantitative analysis of proteome of human nucleoli. Blagoev et. al. performed a temporal analysis of the phosphotyrosine-dependent signaling networks to compare the proteome of three-cell populations. Kratchmarova et. al. analysed the divergent growth factors in mesenchymal stem cell differentiation.

These are just few examples of multiplexed analysis. Now, if you look into literature, there are many studies which have used the SILAC method for comparison of cellular states.

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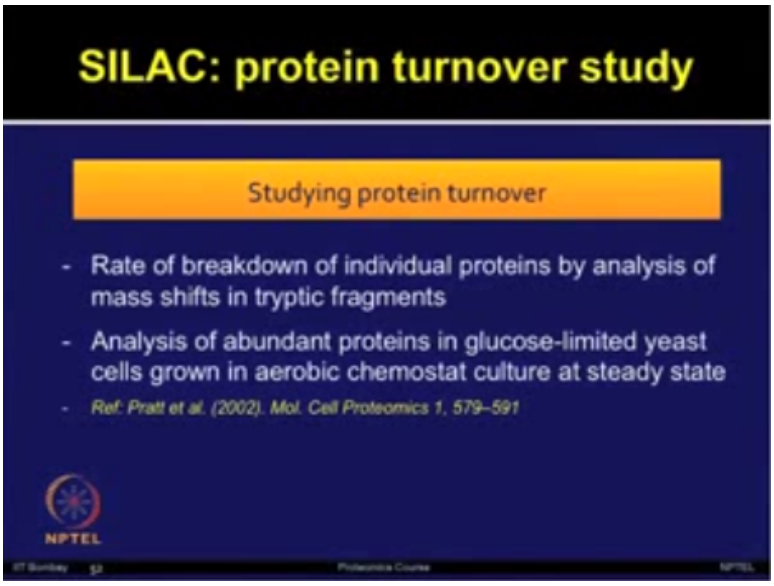

SILAC method has also been used to study the protein turnover. Study by Pratt et. al. used the rate of breakdown of individual proteins by analysis of the mass shift in tryptic peptide fragments. The analysis of the abundant proteins in glucose-limited yeast cells which are grown in the aerobic chemostat cultures at steady state was performed by using SILAC method.

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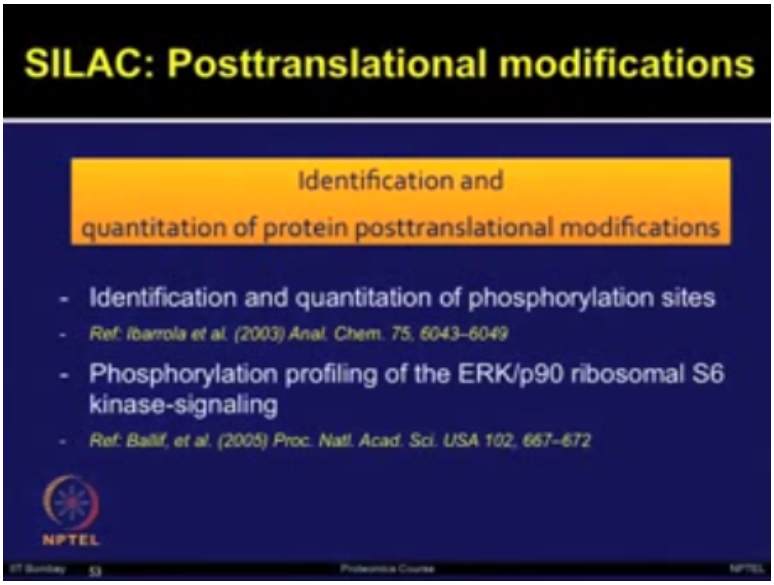

SILAC has been used for identification and quantitation of protein post-translational modification. Study by Ibarrola et. al. identified and quantitated phosphorylation sites. Another study by Bailiff et. al. also identified and quantitated the phosphorylation sites. So, there are many studies which have used SILAC method for studying post-translational modifications.

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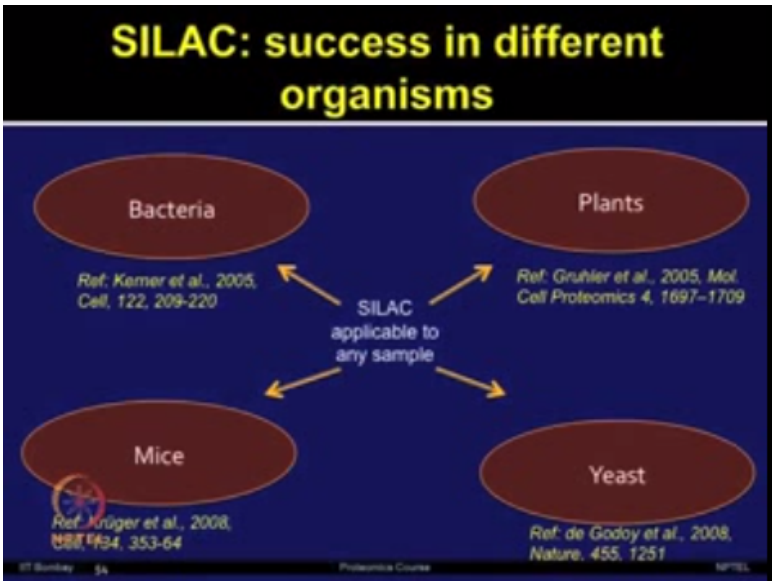

So, interestingly more SILAC method has been used in different organism, in bacteria, in yeast. These were the more commonly used SILAC methods due to the growth in the cell culture but there are some studies on the Arabidopsis in the plants as well as in the mice which has shown that SILAC can be applied to the wide variety of organisms.

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Let us briefly look at the iTRAQ applications and I will show you in this animation.

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In this animation, we will look at one application of iTRAQ method, a study performed by Boyle et. al. in 2010 used the iTRAQ method for identification of candidate biomarkers in ovarian cancer serum.

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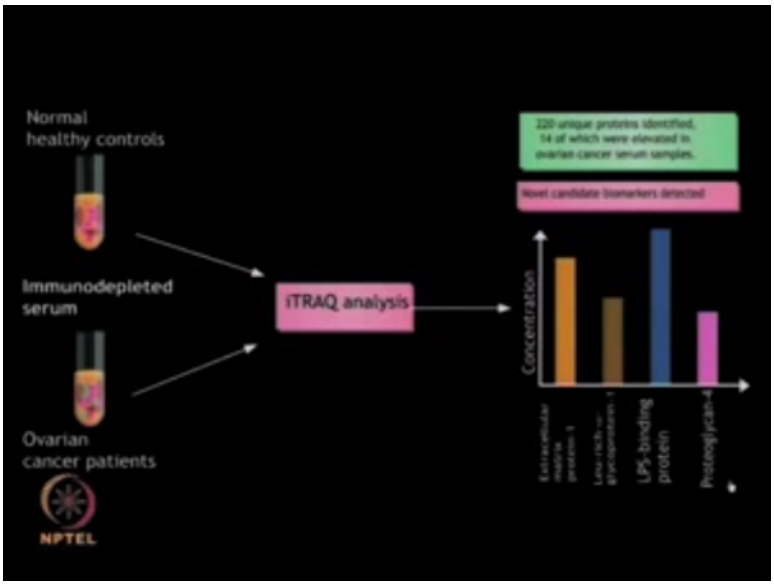

Serum samples of controlled and cancer patients were first of all depleted by using multiple of affinity removal system to carry out immuno depletion of the serum samples from normal controls and ovarian cancer samples. This step helped in removing the high abundance proteins leaving behind only the medium and low abundance protein for iTRAQ analysis. The immuno depleted serum samples were then labelled with iTRAQ regions and further analysed in MS/MS.

The author detected a total of 220 unique proteins of which 14 were found to be elevated in ovarian cancer serum samples as compared to the healthy controls and for normal candidate, biomarker was first-time reported. These results were further validated by the Western immune blotting. This just gives you an overview of how iTRAQ regions can be used for various types of applications including biomarker discovery.

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References

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