LECTURE-19

Applications of 2-DE and DIGE

TRANSCRIPT

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 the previous lecture we started discussing about some case studies about 2-DE applications. So as you have studied in this module what are different type of workflow involved in performing 2-DE experiments. We have gone step by step, starting from protein extraction doing the quantification, isoelectric focusing 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 SDS-PAGE based on the molecular weights of the proteins followed by staining the gels for visualizing the spots. And then scan those images and analyze those images using various software's and then perform statistical analysis to obtain some biological insight. Now, this workflow remains same regardless of whatever application one wants to use in their different types of biological questions. I started discussing about 2-DE applications in the last class, I gave you an overview on 2-DE at that class. So lets continue our lecture from the same theme and let's discuss other case studies how people have employed 2-DE, the power of this technique to resolve thousands of proteins and compare those for various proteomic applications.

So today's lecture outline, first we will continue our case studies on conventional 2-DE. We will talk about the drug treatment on malarial parasite *Plasmodium falciparum* we will then take proteome analysis of SARS virus. After looking at these studies and discussing 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 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 the serum proteome

analysis of prostate cancer patients. So what I want to convey that there are multiple applications one can perform by using this platform and just to overcome certain limitation which are inherent to the conventional 2-DE now people are also applying the 2DE-DIGE approach. But the workflow almost remains the same except the sample preparation part and then this powerful technology can be applied for several applications.

So let's start with the first case study. Towards a proteomic definition of CoArtem action in *P. falciparum* malaria; studied by Makanga *et al.* in 2005. So as you know each year millions of new malaria cases result in over 1 million of deaths worldwide. But due to lack of effective vaccine and widespread of resistance to anti-malarial drugs lot of deaths are happening and the malaria problem is still posing challenges for its control. The anti-malarial therapy of chloroquine and pyrimethamine these have not been able to control the mortality rate because of the anti-malarial drug resistance development. Therefore, there is an urgent requirement for identifying new drug targets as well as understanding the course of action of these drugs by applying various types of high-throughput techniques.

So in this paper, the authors have described how two drugs which are effective for the anti-malarial can be studied for looking at the proteome changes in the *P. 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 authors have applied proteomic approaches, 2-DE to study the proteomic alteration of each of these drugs. So these drugs are applied as drug of choice for all cases of non-severe malaria worldwide. The artemesinin drug action is mediated specifically through its endoperoxide moiety. However more detailed mode of action of these drugs is still unknown.

The purpose of this study was to investigate the action of two active components of CoArtem; artemether and lumefantrine on human malarial parasite *P. falciparum* and

authors tried to look for alterations in parasite proteome, which were induced by each of these drugs. To obtain the insight of proteomic alteration they separated the proteins on the 2-DE gels and compared the response of these proteomic alterations based on these two drugs. And then they identified certain proteins, which were commonly expressed due to these drugs or they were differentially expressed due to these drugs. Certain proteins were found to be commonly up-regulated due to both of these drugs and certain proteins have different patterns.

But before looking at proteomic alterations authors first determined IC10, 50 and 90 values for both the drugs. ARM and LUM we will the abbreviations for Artemesinin and lumefantrine. And effect of these concentrations of drugs on parasite growth over 24 hours was characterized. As you can see the growth curve in the slide, synchronized ring stage parasites were harvested over 24 hours period after the exposure to ARM and LUM. The parasite growth was determined by using Hypoxanthine uptake assay.

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

Once the protein extraction was done then the authors used IPG strip of pH range 3-10 for the first dimension separation of proteins in the linear IPG strips. After IEF was done they equilibrated these IPG strips and then applied that on 12.5% vertical SDS gel. After second dimension separation based on molecular weight these gels were stained with silver or coomassie brilliant blue stains. By employing 2-DE and comparing these gel images by using the PDQuest software authors were able to see that there is a differential proteome response, which is drug specific.

So quantitative analysis of the altered protein expression levels following exposure to ARM and LUM were analyzed and then those were differentially expressed and statistically significant were further analyzed with mass spectrometry based analysis.

The comparative analysis of 2-DE gels from untreated and drug treated protein fractions provided direct and distinct alterations in parasite proteome following ARM or LUM drugs. Certain drugs were identified and 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 proteins, aspartic proteinase and HSP60, 70 and 90 were up-regulated in both the drugs. There are certain proteins such as enolase, fructose biphosphate aldolase and phosphoglycerate kinase were down-regulated in ARM treatment and up-regulated in LUM treatment.

Interestingly, the ARM treatment resulted in more than 3-fold down-regulation of glycolytic enzymes such as enolase, fructose biphosphate aldolase, phosphoglycerate kinase and glyceraldehyde-3-phosphate-dehydrogenase. The expression of same enzymes was also up-regulated more than 3-fold due to the LUM treatment. However certain proteins such as stress response proteins like heat shock proteins which were commonly induced due to either of these drug treatments which looks like general stress response as compared to any unique response to the given drugs.

From this study the major findings were that the authors successfully investigated the alterations of the 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 quiet interesting including a membrane bound calcium binding protein which was up-regulated due to ARM and LUM treatment. The study also established a relationship between the pharmacologically relevant concentration and time of exposure for the two components of CoArtem.

Let us now move on case study 2. Plasma proteome analysis of severe acute respiratory syndrome (SARS); study by Chen *et al*. in 2004.

So the purpose of this study was to perform a comprehensive plasma proteome analysis of SARS and its comparison with the healthy individuals. In this study authors

employed conventional 2-DE, analyzed those images from treatment with 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 blot.

If you remember few years ago SARS occurred in 2002-03 and thousands of deaths were reported in many countries around the globe. The genome sequence of SARS virus was already known and the structure of main protease and receptors were also known. However, the pathogenesis of SARS was not clearly understood. Since serum or plasma provide very valuable sample to identify the targets for diagnostic, prognostic and therapeutics. Authors used plasma sample for analysis of proteomic alteration in the SARS patients.

For the plasma proteomic analysis authors explored the possible pathogenic mechanism of progression of SARS by analyzing plasma proteins of 22 different plasma samples, which were obtained from 4 SARS patients and 6 healthy controls. Authors analyzed plasma proteome by using 2-DE on 4-7 pH IPG strips and they stained the gels with sensitive stain SYPRO Ruby. In the slide you can see the steps involved in the traditional 2-DE followed by mass spectrometry. Both the controls and the treatments; the healthy individuals as well as the patients suffering with SARS, the plasma was separated and then proteins were resolved on 2-DE gels. Differentially expressed proteins were further subjected to in-gel trypsin digestion followed by mass spectrometry.

The comparative proteomic analysis of these 2-D gels revealed that 38 protein spots were differentially expressed more than 2 fold change. Among these spots there were 35 proteins which were up-regulated and 3 were down-regulated. Now these 38 interesting significant and differentially expressed proteins were excised from the gels and subjected to 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 using Mascot search engine.

After the identity of these proteins was established the seven proteins which were not detectable in the healthy controls and only observed in the SARS patients, the identity of those included glutathione peroxidase, PrxII, retinol binding protein, vitamin D binding protein and serum amyloid A proteins. They also found that there are 8 proteins, which are over-expressed and those included pigment epithelium derived factor, 2-HS glycoprotein, complement factor H related protein and leucine rich-2 glycoprotein. For complete list of the proteins identified you can refer to this manuscript. These are some of the interesting proteins authors identified.

After the identity of these proteins was established then western blot analysis was used to validate few targets. Among the identified proteins, authors found that peroxiedoxin II was very interesting. They observed that an intracellular protein PrxII exclusively found in the plasma of SARS patients but was absent in the healthy individuals. These results demonstrated that PrxII can be present in the monomeric or dimeric form. So they performed the western blot in both reducing and non-reducing conditions as you can see in the slide. Then they loaded different samples including recombinant peroxiredoxin, normal or healthy individual patient sample 1 and patient sample 2. So the western blot result showed that the PrxII is present as monomeric form at 22 kDa as you can see the molecular weight mentioned in the slide in the reducing condition and in the dimeric form at 44 kDa under the non-reducing conditions. These results demonstrated that 4 out of 20 SARS probable cases and 4 of the 20 suspected cases showed higher up-regulation of plasma perxoxiredoxin II.

Among various proteins which were interesting identified in this study, peroxiredoxin II appeared quiet interesting and because it was validated by independent techniques. So the level of plasma PrxII in patients with SARS was significantly high. Proteomic analysis and western blot analysis suggested that PrxII may be used as one of the SARS disease associated biomarkers.

They also found one of the several acute phase proteins or APPs those were differentially expressed. The active innate immune responses and oxidation associated

injuries may play a major role in the SARS pathogenesis. The APPs found in the SARS patients suggested that the health status of liver in these patients may be affected their defense against the SARS virus. So some of this information authors were 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 the malaria parasite and other the plasma proteome alteration due to the SARS infection. Now proteomic investigations are quiet are promising but the major limitation of using the conventional 2-DE is if you remember earlier lectures we discussed why some of the inherent limitations of this 2-DE need to be overcome with some more refined techniques. That's where 2D-DIGE comes into the play. So the quantitative protein expression analysis that remains challenging in proteomics. 2-DE is well suited to separate and analyze proteins as well as their isoforms and the optical detection of proteins with the fluorescent tag provides the excellent dynamic range. So can we combine these two powers, one; the conventional 2-DE of separating thousands of proteins and the other sensitive fluorescent dyes which could be highly sensitive. So in 1997 Unlu and colleagues combined these two properties of 2-DE and fluorescent tag and applied this method for DIGE quantitative analysis. So let's look at a case study using 2D-DIGE. But before we touch upon the case studies let me give you an overview of the DIGE technique.

Among 2-DE and DIGE which one of these two techniques will be better to separate serum protein samples obtain from large number of patients in a clinical trial. If you have multiple serum samples from patients, 2-DE although a very useful technique but it may not be the best option in this case to analyze large number serum proteins from large number of patients. In this case DIGE will extremely valuable tool for analysis of large number of samples. Simultaneously without having to overcome the problem of gel-to-gel variations. In DIGE gels controls and test samples can be differentially labeled by using the cyanine dyes then run on the single gel. The pooled internal standard for DIGE is prepared by mixing equal amounts of all the samples that need to be run on the

gel and this prevents the problem of gel-to-gel variations. From the same gel 3 different images can be obtained for Cy2, Cy3 and Cy5. Therefore there will be no reproducibility issue and various artifacts can be removed for the clinical or large number of sample analysis, which we have discussed earlier.

I will just discuss an overview and for more detailed protocol and detailed workflows please refer to previous lectures. So the aim for the development of difference in gel electrophoresis was to overcome the inherently poor reproducibility of conventional 2-DE. So DIGE is quiet sensitive technique with less than 1 fM of proteins, which can be detected and it can enable the linear detection of very broad dynamic range of proteins. So as you can see in the slide the protein samples; lets say we have controls and treatment those are labeled with 2 different dyes Cy3 and Cy5. But a small aliquot of both of the samples is mixed together to make an internal pool.

That internal pool is labeled with another dye Cy2. Now all these protein samples are mixed together in one tube, which contains control and treatment as well as the reference spots from the internal pool. All these protein mixtures are separated in the first dimension on the same strip. And then the same gel can be scanned with the 3 different wavelengths to obtain images from Cy2, Cy3 and Cy5. So in the conventional 2-DE the gel-to-gel variations, which come from the acrylamide polymerization, electrical, pH 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. All those artifacts can be minimized on the DIGE gels. So in the slide it is shown that the 3 samples are mixed and then the isoelectric focusing is performed on the pooled sample. And then this strip is placed on 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. So if your aim is to resolve thousands of proteins and cover comprehensive proteome coverage then DIGE is a very good platform, especially if you

want to do the comparative or differential proteomic analysis. Because your gel-to-gel variations and other variations will be minimized and DIGE will provide high sensitivity.

So once you have run these gels from the same gel 3 images can be obtained and these can be analyzed using various software such as DeCyder software. And then by looking at 3D views and statistical data these proteins can be considered as interesting for further investigations. Once the spots are analyzed and excised from the gels then the same tradition has to be followed you can use any mass spectrometry platform. And obtain the MS spectra for further analysis using various bioinformatics tools such as Mascot.

So overall the DIGE method is far more superior in terms of reproducibility compared to conventional 2-DE and for the quantitative accuracy. Therefore applications of 2D-DIGE can be found in virtually all research areas. If you see in the recent publications you will appreciate there are several papers in each of the biological systems for various types of applications whether at cell signaling, looking at developmental biology, looking at plant proteomic analysis, neurosciences, clinical studies, different types of diseases including cancer. You will find hundreds of publications, which have employed the power of 2D-DIGE techniques.

So let's talk about a new case study, case study 3 on 2D-DIGE as a strategy to identify serum markers for the progression of prostate cancer. This was studied by Byne *et al.* in 2009. In this study authors aim for the identification of serum markers by depicting the progression prostate cancer by using DIGE technique. Prostate cancer is recognized as significant problem in older male population. The prostate cancer screening relies heavily on the testing for the prostate specific antigen or PSA within the peripheral circulations. PSA is a very sensitive marker but there is a lot of discussion on reliability and specificity of PSA on prostate cancer. Reason being that the level of PSA is also high in benign prostatic hyperplasia or prostitis. Therefore, there is lot of discussion whether one should rely on only PSA for the detection of prostate cancer. So this study

aims to identify some new markers in prostate cancer by studying serum proteome analysis.

As you are aware, in fact we have discussed the protein preparation from the serum earlier so each of the biological sample possess lot of technical challenges and serum is one among them where presence of high abundant proteins such as albumin and immunoglobulins they result in 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, anti-trypsin, IgA, transferin and haptoglobin. After the abundant proteins were depleted from the serum samples then the authors moved on for the protein extraction and further analysis.

So the differential proteomic analysis was performed in two different cohorts of histologically confirmed prostate cancer with different grades of the diseases. They used patients with two different grading system based on Gleason grading. So the Gleason grading system that is used help and evaluate for prognosis of men with prostate cancer. Depleted serum samples obtained from 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 labeled with Cy3, Cy5 and also the internal reference pools were made which were labeled with Cy2 dye. These samples were then mixed. The depleted cancer serum from first cohort of Gleason score 5 and second cohort score of Gleason score 7 those were mixed and separated in the first dimension and followed by proteins separated in the second dimension.

So when authors analyzed these DIGE images they found that 63 protein spots were differentially expressed between the Gleason score 5 and Gleason score 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 conventional gels where you have separate gels obtained from each of these groups. But analysis in the DIGE gel is more automated. If you remember our previous

discussion on DIGE gel analysis this analysis is more automated and more straightforward. But you have to go through individual spots and you have to look for how real, how significant those changes are and you have to look at the 3D views of those spots to ensure that it is reproducible among various control and treatment groups. So the different levels of analysis is performed which we have talked earlier but this just shows you the final output that the 63 spots after all the analysis is done were considered significant.

After 2D-DIGE image analysis authors excised those spots and used mass spectrometry to identify those proteins. So the proteins excised from gel were analyzed using mass spectrometer and data from these MS-MS experiments was analyzed using Bioworks browser by using sequest program. Authors also tried the analysis of these DIGE gels by using two different software packages; the DeCyder and Progenesis just so that they are very confidant that all the proteins which they are going to analyze with mass spectrometry are very reproducible. So among the proteins which were common in both DeCyder and Progenesis and the identity of those proteins was further established by using mass spectrometry. Those proteins included pigment epithelium derived factor (PEDF), which was down-regulated in both DeCyder and Progenesis. Zinc-Alpha 2-glycoportein (ZAG) was up-regulated from both the software analyses. Ficolin 3 was down-regulated and Apolipoprotein A-II was up-regulated in both the software analysis. These spots were having similar or uniform trend regardless of what software they analyzed and the authors thought that 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 the validation authors employed various techniques including western blots, ELISA and immunohistochemistry. So the proteins like PEDF and ZAG were further validated by the ELISA technique. The PEDF levels were quantified by using ELISA kit and results demonstrated 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 ZAG ELISA analysis, which is shown in red in the bottom that indicated 1.4

fold increase in ZAG absorbance in the Gleason score 7 group. These studies, these ELISA validations confirmed their findings from the 2D-DIGE experiments.

Authors also employed immuno-histochemistry or IHC for validating the PEDF and ZAG so that they are very confidant that the proteins which they have identified from proteomic profiling those are real and also tested 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. Authors confirmed that differential expression of PEDF and ZAG could be performed using various validation techniques such as western blot, ELISA and immunohistochemistry. Based on their studies and follow up experiments they concluded that PEDF could be a potential marker of early stage prostate cancer prediction. However, more studies and follow-up required on large number of patients before it can be established as a good biomarker.

In summary in today's lecture we finished 2 case studies on 2-DE, which were continued from the previous lecture. We discussed about different types of drug treatment in malaria parasite *P. falciparum*. We also discussed about SARS proteome alteration in the plasma due to the SARS infection. And after looking at these two conventional proteomic analysis by using 2-DE 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 case study of prostate cancer, the serum proteome analysis. The one thing which you will observe that all the middle steps in the entire workflow of 2-DE and DIGE are very similar. It's the very first step, the sample preparation, which is quiet distinct in each of these techniques and quiet unique for various types of biological samples. All your creativity and your modifications play major role during the first step. Once you have obtained good protein abstract then same protein extract can be employed for various types of technologies and then the workflow of all the steps we talked about earlier for 2-DE and DIGE that remains very similar. So in that regard you would not have noticed

too much difference in the overall applications, whether we have talked about plasma proteome analysis based on the conventional 2-DE or serum proteome analysis of prostate cancer based on DIGE technique because at the end you want to establish the identity of proteins by using mass spectrometry approaches. But how many proteins' identity you can actually establish that fact is the major discriminator between two techniques. You might lose lot of significant spots by using only conventional proteomic analysis. Because you have to apply large number of gel for comparisons. But regardless of the pitfalls of each of the techniques you may appreciate that there is lot of power of these techniques and these can be employed for any type of biological applications. You pick an application of your choice and I am sure you will be able to answer those by employing 2-DE or 2D-DIGE techniques. Thank you!