LECTURE-8

2D-DIGE CLINICAL APPLICATIONS

HANDOUT

PREAMBLE

Two Dimensional Electrophoresis (2DE) continues to be one of the most commonly used techniques for proteomic studies. The technique mainly involves the separation of proteins on the basis of their isoelectric point and subsequently on the basis of their molecular weight. The Difference in Gel Electrophoresis (DIGE) technique, which presents advantages over the conventional 2 DE is now widely used. The advent of DIGE has reduced post-electrophoretic processing and the amount of initial protein sample required for the run. More importantly, since DIGE employs fluorescent dye labeling of the sample and hence different samples can be run on the same gel, it ensures that the phenomenon of gel-to-gel variability is eliminated. 2D-DIGE can encompass a wide variety of applications in proteomics, including deciphering the differences in protein expression pattern under the influence of different set of conditions, biomarker discovery, post-translational modifications, understanding the composition of a complex mixture of proteins etc. These individual facets can be extrapolated or amalgamated towards clinical applications.

OUTLINE OF LECTURE

1. Overview of the steps involved in 2DE

2. Tabular depiction of clinical applications

3. Case study for 2DE

4. Case study for DIGE

5. Conclusions

BOX-1: TERMINOLOGIES

- **2-DE:** Two Dimensional Electrophoresis, brings about separation of proteins on the basis of their isoelectric point in the first dimension, which is followed by their separation on the basis of their molecular weight in the second dimension.
- **DIGE:** Difference in Gel Electrophoresis, which is a modification of 2DE, in that it has the individual protein samples labeled with fluorescent dyes and two different samples and an internal control which itself is labeled with a third fluorescent dye can be all visualized in a single gel.
- **Internal control in DIGE:** It is prepared by mixing equal amount of all the samples that need to be run on the gel.
- **Proteome:** The entire complement of proteins expressed by the genome of an organism under specific defined conditions is known as the proteome. Like the transcriptome, the proteome of an organism will also vary with external factors and conditions.

1. AN OVERVIEW OF THE STEPS INVOLVED IN 2DE

WORKFLOW

1. Isoelectric focusing

2. Equilibration of IPG strips

3. SDS-PAGE (second dimension)

4. Staining – gel visualization

5. Image analysis

6. Spot picking

7. Enzymatic digestion

8. MS Analysis

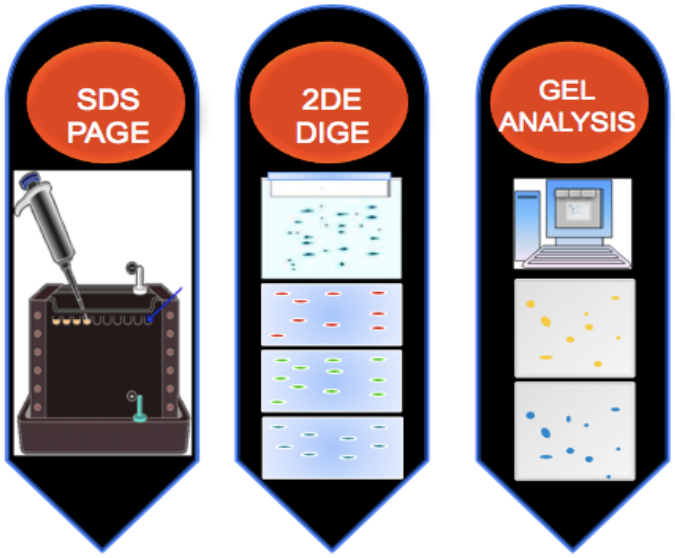

Fig. 1. An overview of gel-based proteomic techniques

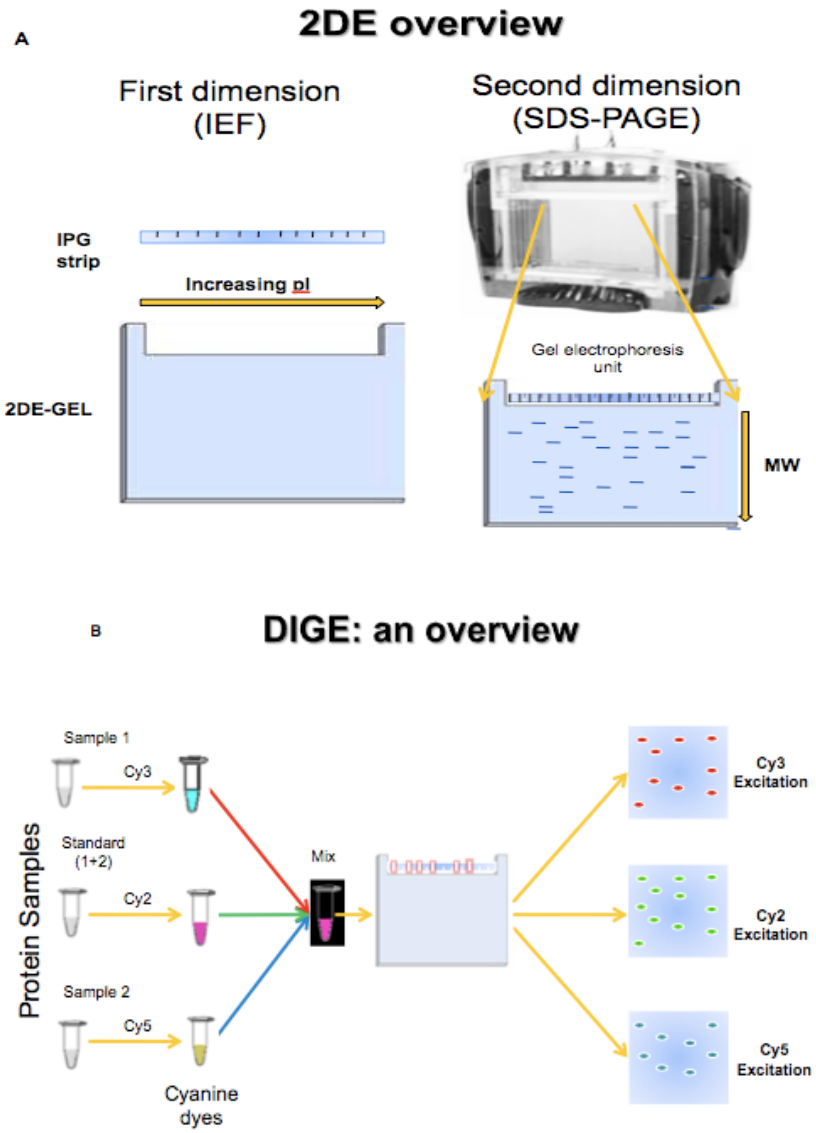

Fig. 2. Depiction of typical 2DE (A) and DIGE (B) techniques

DIGE differs from 2DE in that it involves the protein samples being fluorescently labeled and the gels are scanned at different wavelengths, which correspond to the excitation wavelength of the individual dyes and hence different fluorescence patterns can be

established. The images are then analyzed further using softwares like Image Master Platinum (For 2DE) or DeCyder (For DIGE). The significant spots can be further selected for protein identification by Mass Spectrometry.

2. CLINICAL APPLICATIONS OF 2DE AND DIGE: TABULAR DEPICTION

TECHNIQUE	SAMPLE USED	KEY FINDIINGS	REFERENCE
2DE	Human serum	Identified 20 differentially expressed proteins in osteoarthritis patients as compared to healthy patients	*Carolina Fernandez et al. Proteome Science 2012.*
	Carcinoma tissues of human bronchial epithelia	Proteins related to carcinogenesis of bronchial epithelia were identified	*Wu xy et al. Chinese Journal of Cancer 2004*
	Bacteria	Identified proteins with altered expression under the influence of oxidative stress in *P. intermedia* spp.	*Santos G et al. Anaerobe 2012*
	Human serum	Characterized six proteins which exhibit differential expression patterns in falciparum and vivax malaria and hence could be used as potential biomarkers for malaria detection.	*Ray S. Et al. Plos One 2012*
	Sera of Gastric cancer affected	Novel candidate markers for human gastric cancer	*Pennoma et al. Journal of*

DIGE	patients	were identified.	*Proteomics. 2012*
	Oral Mucosa of HIV infected patients	Differentially expressed proteins in HIV-infected HAART subjects in comparison with healthy controls	*Yohannes et al. Plos One. 2011*
	Glomeruli of mice with distinct nephropathies	Identification of protein biomarkers for glomerular alterations	*Blutke et al. Proteomics Clinical Applications. 2011*
	Human brain synaptosomal preparations from humans with chronic alcohol abuse	Identification of Proteins associated with synaptic activity and their alterations due to alcohol abuse	*Etheridgen et al. Proteomics Clinical Applications. 2009*

Illustration: Serum proteome analysis using gel-based proteomics

Steps involved in performing serum proteome analysis are demonstrated in following interactive animation.

Illustration: Bacterial proteome analysis using gel-based proteomics

Steps involved in performing bacterial proteome analysis are demonstrated in following interactive animation.

3. CASE STUDY FOR 2DE

Plasma samples are widely analyzed as identified proteins could be used for diagnostic, prognostic and therapeutic applications. In this study, comparative proteomic studies on the plasma from SARS-virus infected patients and healthy controls were carried out using techniques like 2DE in combination with MALDI-TOF/TOF and LC-MS/MS for identification of differentially expressed proteins. The identified proteins were validated by Western Blot analysis for the same samples.

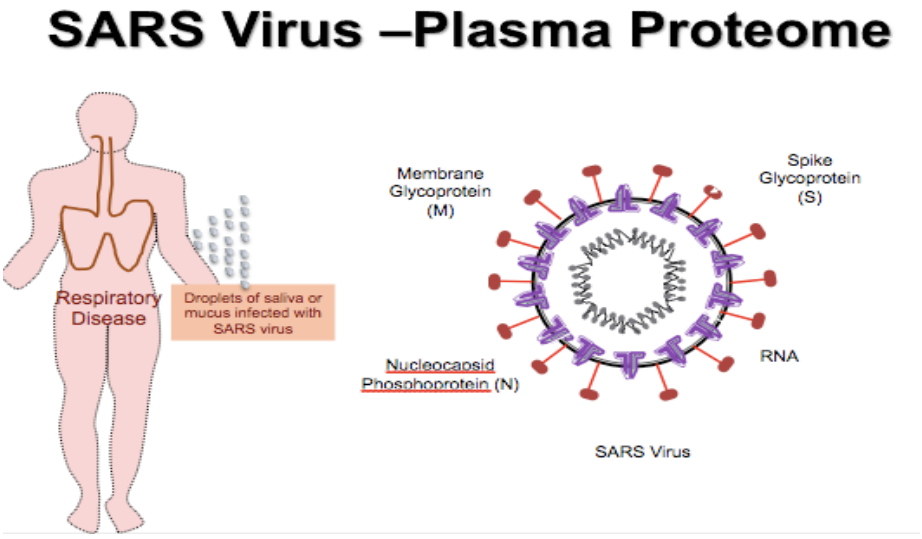

Fig. 3. Plasma proteome analysis of severe acute respiratory syndrome (SARS), study by Chen *et al*. in 2004.

WORKFLOW ADOPTED FOR THE STUDY:

1. 22 different plasma samples were obtained from 4 SARS patients and 6 healthy controls.

2. Plasma samples were analyzed by 2-DE on 4-7 pH IPG strips in the first dimension.

3. This was followed by second dimension SDS-PAGE and gel-staining using the sensitive stain SYPRO Ruby.

4. 38 Proteins were found to be significant because of their differential expression and were further subjected to Mass Spectrometry for identification, and the data was analyzed using the Mascot search engine.

5. Seven proteins which were only observed in the SARS patients included glutathione peroxidase, PrxII, retinol binding protein, vitamin D binding protein and serum amyloid A proteins. 8 proteins over-expressed in SARS patients included pigment epithelium derived factor, 2-HS glycoprotein, complement factor H-related protein and leucine-rich Alpha-2 glycoprotein.

6. After the identity of these proteins was established, the western blot analysis was used to validate a few targets.

7. Intracellular protein PrxII (secreted by T cells) was exclusively found in the plasma of SARS patients but was absent in the healthy individuals. Western blot analysis revealed that PrxII is present as monomeric form at 22 kDa under reducing conditions while is present in the dimeric form at 44 kDa under non-reducing conditions.

8. Overall, this study has helped reveal an initial profile of alterations in plasma proteins of SARS patients, and the identification of PrxII as a potential candidate for SARS biomarker.

4. CASE STUDY FOR DIGE

This study employed use of 2D-DIGE for investigation of biomarkers for the identification of early prostate cancer. Though Prostate Specific Antigen (PSA) has been extensively defined as a very sensitive marker for prostate cancer; its reliability and specificity is in question. This is mainly because PSA is also found to be elevated in patients suffering from prostatic hyperplasia or prostitis. This can further pose serious consequences as the disease can be wrongly diagnosed and eventually treated wrongly. This makes it necessary to develop a marker that exhibits improved efficacy of early screening of prostatic cancer.

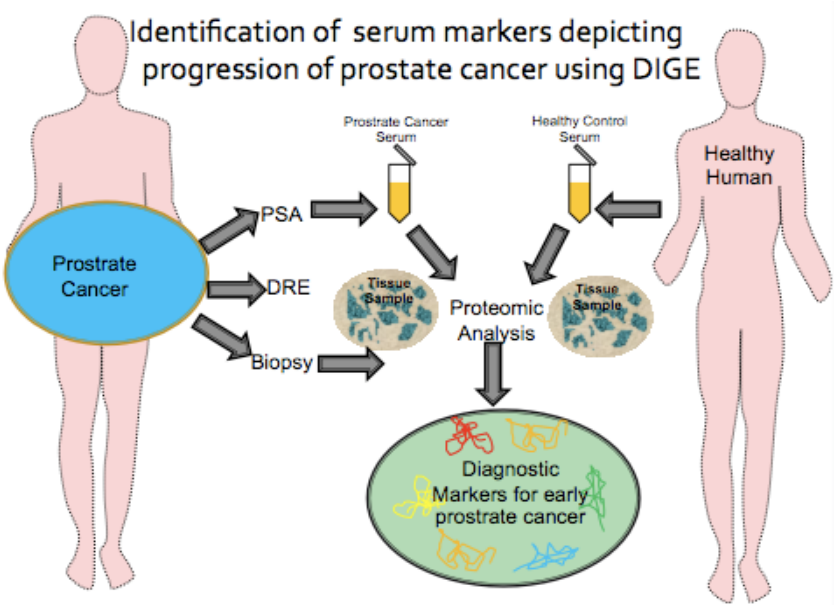

Fig. 4. Identification of serum markers for progression of prostate cancer (study by Byrne *et al.* in 2009)

WORKFLOW ADOPTED FOR THE STUDY:

1. Serum samples were obtained from prostate cancer patients, belonging to different grades as classified according to the Gleason grading system and were divided into 2 cohorts.

2. The abundant proteins in the serum need to be depleted as they can pose as serious artifacts in the downstream 2DE steps. Serum depletion was done using multiple affinity removal system to eliminate albumin, IgG, anti-trypsin, IgA, transferin and haptoglobin.

3. Differential proteomic analysis was then performed on two cohorts of samples by labeling them with Cy3 and Cy5 respectively and an internal control pool was created which contained equal amounts of both the samples and was in turn labeled with Cy2 dye. Both the samples were then mixed and run on a single gel for both the dimensions of DIGE. The gels were then scanned at excitation wavelengths of each of the dyes and the images so generated were analyzed using software like DeCyder and Progenesis.

4. Thirteen differentially expressed genes were found to be statistically significant and were analyzed on Mass Spectrometry for identification of those proteins with the aid of Bioworks browser and Sequest program. Down-regulated proteins included pigment epithelium derived factor (PEDF) and Ficolin 3, while up-regulated proteins included Zinc-Alpha 2-glycoportein (ZAG) Apolipoprotein A-II. Importantly, the same trend was observed for each of these proteins regardless of the software used.

5. The results were further validated using the ELISA technique; Western Blots and Immunohistochemistry to increase the confidence that the proteins identified from proteomic profiling represent relevant biomarkers.

6. Overall, this study has identified PEDF as a potential marker of early stage prostate cancer prediction.

Illustration: Interactivity - Comparison of 2DE and DIGE for clinical studies

Which of these two would be the better technique to separate serum protein samples obtained from 250 patients in a clinical trial?

2-DE, although a very useful technique, may not be the best option in this case for analyzing serum proteins from a large number of patients as it would involve running several individual gels which would be a time consuming process. Also variations across the gels would make comparison of results a problem.

DIGE is an extremely valuable tool for analysis of a large number of samples simultaneously without having to overcome the problem of gel-to-gel variation. The control and test samples can be differentially labelled using the cyanine dyes and run on a single gel.

5. CONCLUSION

This lecture covers few applications of both 2-DE and DIGE. An important facet, which stands to be common to both the techniques, is the workflow, which differs only at the stage of sample labeling and the post-electrophoretic processing. 2-DE or DIGE experiments can be planned depending on the objective of the study, availability of resources and the final application for which it has to be used. An experiment when designed with the right perspective can be of great importance and it can be applied widely in proteomic studies.

6. REFERENCES

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