LECTURE-15

iTRAQ Clinical Applications

HANDOUT

PREAMBLE

Isobaric Tagging for Relative and Absolute quantitation (iTRAQ) is a quantitative MS based method for quantifying proteins subject to various different conditions. iTRAQ involves tagging peptides with a reporter group, and these reporter ions are used for quantitation during mass spectrometry analysis. Due to the sensitivity and ability for multiplexing of iTRAQ, it is widely used for clinical proteomic analysis. The ability to multiplex, i.e., analyze more than two samples at a time, makes iTRAQ an attractive technique for studying various diseases. Based on same principle, iTRAQ can also be used to monitor the effect of a particular protein, for example, effect of some drugs and changes in the level of protein expression. Besides these, iTRAQ are also being considered for studies of post-translational modifications as well as for signaling. Various applications of iTRAQ are discussed in this lecture.

OUTLINE OF LECTURE

1. iTRAQ – An overview
2. Working protocol of iTRAQ
3. Applications of iTRAQ in clinical proteomics
    a) Tumor progression
    b) Biomarker discovery

PROF. SANJEEVA SRIVASTAVA
DEPARTMENT OF BIOSCIENCES & BIOENGINEERING
IIT BOMBAY, MUMBAI, INDIA

c) Comparison of cellular states

4. Conclusion

BOX FOR TERMINOLOGY

Isobaric Tag for Relative & Absolute Quantification (iTRAQ): is a MS-based technique for relative and absolute quantification of proteins present in up to four cell preparations by making use of four isobaric tags that label the proteins via their N-terminal.

iTRAQ label: The iTRAQ reagent consists of a protein reactive group that labels the N-terminus of all peptides as well as free amine groups of lysine side chains, a neutral balance portion and a reporter group, giving it a total mass of 145. The different distribution of isotopes between the reporter and balance groups makes the labels isobaric and enables their detection upon fragmentation and release in MS.

Isotope Coded Affinity tagging (ICAT): A technique used to quantify proteins by labeling cysteine residues with a tag.

Stable Isotope labeling of Amino Acids in Cell culture (SILAC): A technique of labeling proteins by providing tagged amino acids in cell culture, so that the cell take them up and label the proteins metabolically.

1. iTRAQ – AN OVERVIEW

Isobaric Tagging for Relative and Absolute Quantitation (iTRAQ) is a MS-based quantitative proteomic approach for identifying and quantifying proteins from various sources. The iTRAQ reagent is used to label the proteins with a reporter tag, which gets dissociated during fragmentation. The intensity of the reporter tag is a measure of the amount of protein present in the mixture (Fig 1).

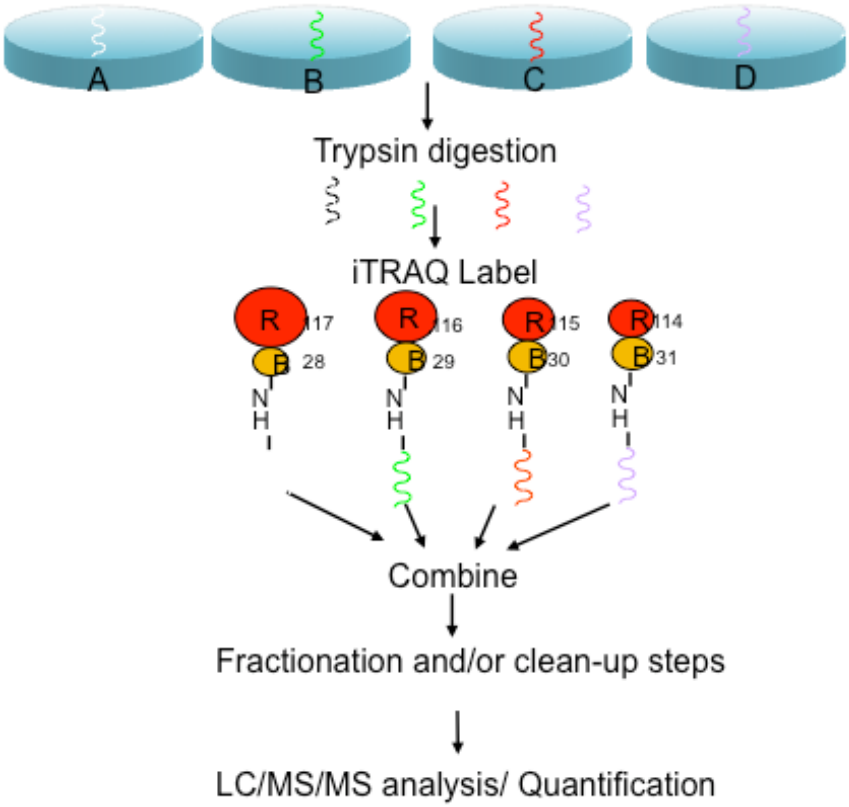

Fig 1. An overview of iTRAQ

One of the biggest advantages of ITRAQ is the ability to handle more than two different samples at a time, i.e. the ability to multiplex. This can be done due to the availability of eight different ITRAQ reagents, which differ from each other by a mass of 1 Da each. The mass of reagents vary at the level of the reporter group, but the overall mass of the

reagent remains the same, due to the balancer group. When handling four or eight different samples, different reporter ion peaks corresponding to the same peptide is obtained. The ratio of the intensity of 4 or 8 peaks is a measure of the amount of that particular protein in all the samples.

2. WORKING PROTOCOL OF ITRAQ

ITRAQ ultimately involves analysis of the protein samples using mass spectrometry. Hence, the samples need to be free from any type of contamination, especially salt. For this purpose, the proteins are first dissolved in an appropriate MS compatible buffer like ammonium bicarbonate, pH 8.5 and then zip tipped by passing through C-18 column to remove excess salt. After eluting the proteins, they are trypsinized overnight and then labeled using ITRAQ reagent. The samples from different sources are pooled together (Fig 2). An additional enrichment step for peptides using reverse phase chromatography followed by strong cation exchange chromatography is usually done prior to MS analysis.

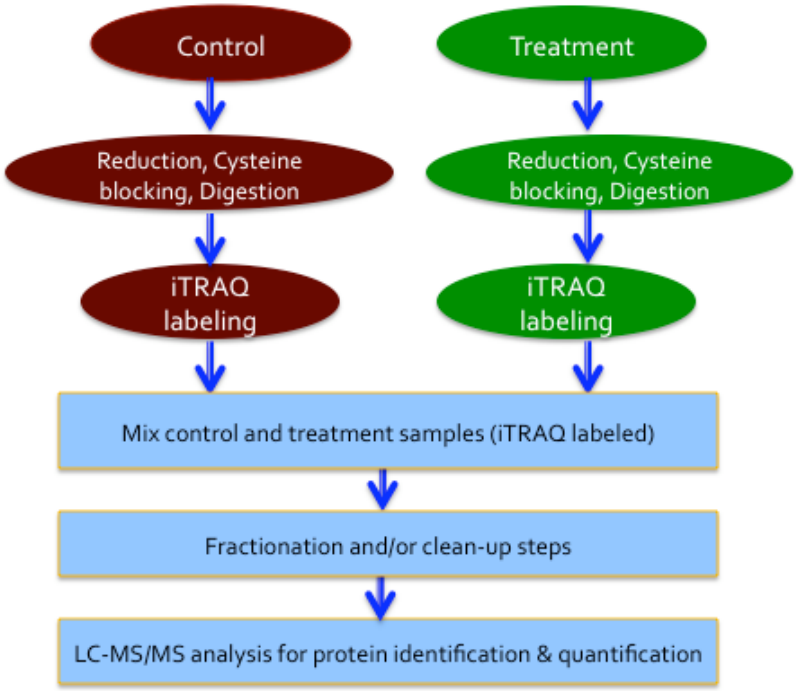

Fig 2. A schematic representation of the entire iTRAQ workflow

3. APPLICATIONS OF iTRAQ IN CLINICAL PROTEOMICS

a. TUMOUR PROGRESSION

Tumor progression is facilitated by many factors, which include increased secretion of growth factors, angiogenesis and increased secretion of proteases and down-regulation of extracellular matrix proteins. iTRAQ based quantitative proteomic approach may be used in this respect to study the progression of tumors with time. After the knowledge of potential biomarkers or target proteins of the particular tumor, their dynamic change in concentration over a particular time interval can be monitored as a method to investigate the progression of tumor. A study conducted by Yixuan et al, on gastric cancer cell lines, showed the increased secretion of Cystatin B and Cathepsin S, a cysteine lysosomal protease, which is responsible for the increased turnover number of many extracellular matrix proteins. Due to the loss of extracellular matrix protein, the tumor becomes invasive. iTRAQ based quantitative approach validated the up-regulation of Cathepsin S in cancer cell lines like MKN7 and MKN 45.

Tumor regression is another aspect of tumor studies that can be studied using iTRAQ. Tumor regression is usually assisted with the death of the tumor cells. Hence, the use of iTRAQ in quantifying apoptosis promoting proteins, like caspases, on treatment with certain drugs, is quite logical. With the same idea in mind, Leong et al studied the effect of Doxorubicin and death receptor ligand, TRAIL on breast tumor regression, by studying the proteomics of mitochondrial and endoplasmic reticulum fractions of breast cancer cells. The efficacy of drug was tested by measuring the quantity of caspase 3 in the mitochondrial fractions. The best result was obtained for

cells treated with10 uM Doxorubicin followed by 5 nM TRAIL for a period of 2 hours. Validation of results was performed by immunofluorescence. The study was conducted using two breast cancer cell lines, three differentially expressed proteins were identified common to both the cell lines, namely, AHNAK, SLC1A5 and PPIB, of which AHNAK and SLC1A5 were associated with cell signaling and both were down-regulated. Therefore, iTRAQ can be used not only to differentiate the various stages of cancer but also monitor their regression due to the drug treatment.

b. BIOMARKER DISCOVERY

Biomarkers are bio-molecules, usually proteins, which serve as identifying agents for many diseases. Hence, biomarker discovery is an extremely hot area for research, especially in cancer diagnosis. However, biomarker discovery is extremely challenging, given the fact that they are extremely low in amount, during the early stage of the disease, which is why sophisticated techniques are required for their detection. Also, because of their low molecular weight, they are often masked by high molecular weight proteins. The introduction of sophisticated and sensitive labeling strategies like iTRAQ and SILAC has revolutionized the usage of MS in biomarker discovery. Using iTRAQ reagent, many important biomarkers have been brought to light.

An example is orosomucoid 2 (ORM2), a potential biomarker for colorectal cancer, which is up-regulated by approximately 4.1 folds during the disease. Zhang et al, reported the increase in the level of ORM2, in colorectal cancer. ORM2 is 24 kDa inflammatory protein present in the plasma in very low amount during the early stages of colorectal cancer. As the disease progresses, the level of ORM2 increases and hence, this protein can be used as a potential biomarker for colorectal cancer. ORM2 because of its low molecular mass is often masked by other serum proteins, and hence, Zhang et al, used depletion approach, where the plasma was depleted of high abundant protein and then using sophisticated micro Q-TOF coupled with ITRAQ, the levels of ORM2 could be detected with a confidence limit of greater than 95%. The ability to quantitate thus provides an idea as to the identity of the potential biomarkers for that particular disease.

A parallel study conducted by Chen et al on urinary bladder cancer led to the discovery of apolipoprotein A I/II, heparin cofactor II precursor as potential biomarkers for the disease. Using iTRAQ based quantitative proteomic approach on urine samples obtained from healthy and diseased individuals, these biomarkers were discovered, which were up-regulated by more than 2 folds. Further, validation was performed using western blotting and ELISA based assays. Another study by Boylan et al. investigated biomarkers in ovarian cancer (Fig 3), which is illustrated in animation.

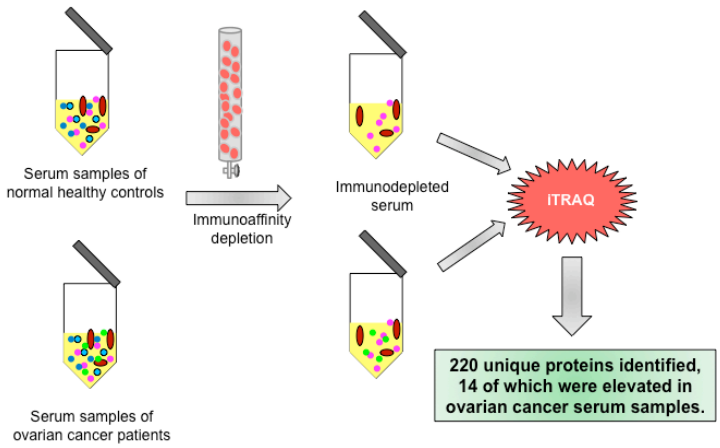

Fig 3. Overview of ovarian cancer biomarker investigation using iTRAQ

Illustration: Application of iTRAQ for ovarian cancer biomarker discovery

A multiple affinity removal system was used to carry out immunodepletion of the serum samples from normal controls as well as ovarian cancer patients. This helped in removing the high abundance proteins, leaving behind only the medium and low abundance proteins for iTRAQ analysis.

The immunodepleted serum samples were then labeled with the iTRAQ reagent and analyzed. The authors detected a total of 220 unique proteins of which 14 were found to be elevated in the ovarian cancer serum samples compared to the healthy controls and four novel candidate biomarkers were detected. Results were validated by Western immunoblotting.

ITRAQ can also be used to validate the proteomic data to that obtained from genomic microarray. For example, iTRAQ based quantitative proteomic approach was used to validate the microarray data and identify novel biomarkers for the disease Tuberculous meningitis, an extra-pulmonary disease caused by *Mycobacterium tuberculosis*. Using ITRAQ based approach, novel biomarkers like amphiphysin and neurofascin were identified, which were validated using immunohistochemistry. ITRAQ, thus provides targets for quick and sensitive detection of several diseases. However, it should be noted that before it can be termed as a "biomarker", the protein need to be validated in large clinical cohort.

Even in Parkinson's disease, iTRAQ found its application in biomarker discovery. It is known that Parkinson's disease almost increases the chances of getting dementia by six fold. An attempt was hence made by Lehnert et al to find potential biomarkers for Parkinson's disease induced dementia (PDD). Using iTRAQ based method for quantifying proteins from the cerebrospinal fluid, biomarkers like Tyrosine-kinase-non-receptor-type 13 and Netrin-G1 were identified against non-demented controls. These were further validated using multiple reaction monitoring, a technique of MS, where the precursor ion is studied. In this case, due to unavailability of suitable antibodies for quantification, synthetic peptides were synthesized corresponding to these proteins, diluted to certain range and then the actual protein concentration from cerebrospinal fluid was calculated from the standard curve.

c. COMPARISON OF CELLULAR STATES

Comparing various cellular states or the lineages of a particular cell type may be considered as another important application of iTRAQ. It becomes extremely important in understanding the origin of a particular cell type, not only in diagnosis but also in the current stem cell based therapy. In stem cell based therapy, stem cells are tuned in, using appropriate cocktail of growth factors and gene expressions, to produce a particular lineage of cells. Understanding the basic biology of that particular cell type using proteomic approach is an important area of research.

In this aspect Onnerfjord et al, studied proteomics of eight cartilaginous tissues using iTRAQ method and identified unique characteristic proteins. Even within the cell, organellar proteomics is a field yet to be explored. An attempt to characterize the proteome of peroxisome in mouse liver at different time points of the life cycle was performed by Amelina et al. The results suggested that approximately eight proteins are differentially regulated with age, majority in the peroxisome. Peroxisome is associated with detoxification of ROS (reactive oxygen species) and with age, as the lysosomal enzymes, capable of detoxifying majority of ROS gets depleted, peroxisome and mitochondria become the last resort of the cells for survival.

4. CONCLUSION

iTRAQ is currently one of the most reliable quantitative proteomics technique. Because of its ease, multiplexity and accuracy of absolute quantitation, it is used in the field of quantitative proteomics, not only in the domain of clinical proteomics, but also extended to microbial proteomics, plant proteomics etc. In clinical proteomic field, its contribution is noteworthy especially in cancer diagnosis and biomarker discovery. ICAT emerged, as the first tool of quantitative proteomics; however, ITRAQ has emerged as very popular quantitative proteomics technique despite powerful competitors like SILAC and protein microarray.

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