SYNOPSIS

DIFFERENTIAL PROTEOMICS OF HEMATOLOGICAL DISORDERS

INTRODUCTION

The HbE-β is one of the commonest forms of hemoglobinopathies worldwide (1). The HbE mutation is located near the junction between the first exon and the first intron of the β-chain gene. Nucleotide sequence change near the consensus splice site region activates a cryptic splice site, which is not normally used for mRNA processing. This new splice site competes with the normal splice site. Some mRNAs are still processed using the normal splice site and thus produce a protein with a Lys instead of a Glu at position 26. The variant (HbE) is thus innocuous in its homozygous states (2). The primary clinical importance of HbE trait arises when the βE allele interacts with other β-thalassaemia mutations leading to a moderate to severe anemia known as HbEβ-thalassaemia (3).

Although the spectra of clinical pathophysiology vary depending on coinheritance of other genetic modifiers, the underlying pathology among the types of thalassemia is similar. This pathology is characterized by decreased Hb production and erythrocytes survival. The excess of unaffected globin chain, which form unstable homotetramers that precipitate as inclusion bodies causes marked erythrocytes damage and severe hemolysis associated with ineffective erythropoiesis (IE) and extramedullary hemolysis (4).

The only definitive form of treatment for thalassaemia is bone marrow transplantation, which is possible only when there is a matching donor relative. Symptomatic treatment involves regular blood transfusion and the use of iron-chelating drugs to remove the excess iron that results from transfused blood. Hence more studies are required to frame a better
disease management program. Therefore a lot of attention being given to the disease monitoring as this is of prime importance for the welfare of the patients.

**OBJECTIVE**

Our objective has been to initiate proteomics studies of the body fluids such as plasma and urine keeping in mind the present disease management system. We have also done lipidomics study of plasma, erythrocytes and erythrocytes membrane. The objectives and scope of the present study would be elaborated in the following three Chapters:

1. **Chapter 1: Plasma Proteomics of Eβ Thalassemia** - conducting a 2D gel electrophoresis (2DGE) based study of the differential expression of proteins of Eβ thalassemic samples as compared to normal samples.

2. **Chapter 2: Urinary Proteomics of Eβ Thalassemia** - 2DGE based study of the changes in the urine proteome of the Eβ thalassemic samples as compared to normal.

3. **Chapter 3: Lipidomics Study of Eβ Thalassemia** - Study the lipidome of plasma, erythrocytes and erythrocytes membrane fractions and analyze the changes in lipidome of the Eβ thalassemia patients as compared to normal samples. We also aim to analyze the levels of oxidized lipids as oxidative stress is a well known condition in Eβ thalassemia.

**Appendix: Urinary Proteomics of Urothelial Neoplasm:** We’ve also initiated study to find changes in urinary proteome of patients suffering from urothelial neoplasm which are classified based on their p53 immunohistochmistry (IHC).

**METHODS**

**Sample preparation**

Plasma was separated from the 2ml of blood sample collected from normal as well as Eβ thalassemic patients using self forming 75% percoll gradient. This plasma fraction was then subjected to 20% ammonium sulphate precipitation to deplete the high abundant
proteins\textsuperscript{(5)}. The precipitate was dialysed and dissolved in 2D rehydration buffer after protein estimation. This was then used to run 2 DGE.

Urine samples (Eβ thalassmeic, urothelial neoplasm and normal) (~30ml) were centrifuged to remove cellular debris \textsuperscript{(6)}. The supernatant was then centrifuged in Amicon ultra centrifugal filter units with 5kDa cut off membrane concentrated to a volume of 2ml and proteins were precipitated using 75% ethanol. The precipitate was directly solubilised in 2D rehydration buffer and stored for further analysis.

The plasma and erythrocytes were separated according to density using 75% percoll. A fraction of the erythrocytes was further lysed and the erythrocyte membranes were taken separately. Lipid extraction was done using methyl tertiary butyl ether (MTBE) as described in \textsuperscript{(7)}. The samples were then vortexed for 1 hour and subsequently centrifuged. 800 µL of the upper organic phase was transferred into a new vial and stored at -20°C until analysis.

\textbf{2DGE, mass spectrometry and western blot analysis}

The solubilised samples (plasma and urine) were separated first on the basis on pI on 17cm pH 3-10 IPG strips and then on the basis of molecular weight by 2DGE. Gels were stained either with colloidal Coomassie \textsuperscript{(8)} or sypro ruby according to manufacturer’s instructions. Densitometry analyses were done on Versa Doc series 3000 imaging system using PDQuest software (version 7.1, Bio -Rad). Spot volumes (intensity) of the desired spots were normalized as parts per million (ppm) of the total spot volume in gels, to calculate the relative abundance of a spot in a sample. The protein spots from 2D gels of normal as well as diseased samples were excised, and annotated using MALDI ToF/ToF (AB 4700, Applied Biosystems) following published protocol \textsuperscript{(9)}.

Few differentially regulated proteins were further analysed by western blot analysis and student t-test performed. For plasma β tubulin was used as loading control. In the absence of
any proper loading control, in case of urine samples, we have stained the total blot and the intensity of each band is normalised against the total intensity of the lane.

Mass spectrometry of lipids

For mass spectrometric analysis, dilutions were made of the extract with CHCl₃/MeOH/2-propanol 1/2/4 (v/v/v) containing 7.5 Mm ammonium acetate. Mass spectrometric analysis was performed on a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a robotic nanoflow ion source using chips with 4.1 mm nozzle diameter. The ion source was controlled by chipsoft 6.4. software (Advion BioSciences) and operated at the ionization voltage of 0.95 kV and gas pressure 1.25 psi. MS survey scans were acquired in positive and negative ion mode using the Orbitrap analyzer operated under the target mass resolution of 100,000 FWHM (Full Width at Half Maximum). Targeted MSⁿ experiments were performed using pulsed Q-dissociation (PQD) for positive ion mode and high energy collisional dissociation (HCD) for negative ion mode using the LTQ Orbitrap machine.

The raw data files acquired were converted to *.mzXML format using MS converter from ProteoWizard. Mass spectra were further processed by Lipidxplorer software and lipids annotated by matching the m/z of their monoisotopic peaks to the corresponding elemental composition constraints using molecular fragmentation query language (mfql). The mfql for around 19 major lipid classes were used. To analyse the oxidized species hydroxyl, keto, hydroperoxide, epoxy and polyhydroxy derivatives of PC as long chain oxidized PCs and saturated or unsaturated aldehyde and carboxylic acid derivatives of truncated PCs were only considered. Hierarchical clustering (single linkage) study was done on the entire data set of the three fractions separately using Cluster 3.0. The similarity protein expressions data was measured by correlation (centered). Further PCA was applied to
the peak lists produced from all samples analyzed in each fraction separately using XLSTAT (version 2014.3.01) software.

RESULTS AND DISCUSSIONS

1. Plasma Proteomics of Eβ Thalassemia

Figure 1: Representative gels of the four categories analysed. A - Normal sample, B - Eβ thalassemic sample, C - EE homozygous sample and D - high HbF percent without any diseased condition.

1 = Transferrin; 2 = alpha 1 antitrypsin; 3 = plasminogen; 4 = apolipoprotein A IV precursor; 5 = haptoglobin precursor; 6 = alpha 1 microglobulin/ inter alpha trypsin inhibitor precursor; 7 = vitronectin; 8 = adenylate kinase 1; 9 = apolipoprotein A IV; 10 = apolipoprotein A I; 11 = glutathione S transferase A2.

We have further compared these changes with plasma samples of homozygous EE condition as well as with another non-thalassemic condition where the patient exhibits high level of fetal haemoglobin (HbF).
Figure 2: A bar plot representing the changes in the protein levels in the four categories. Significant (p ≤ 0.05) results are marked by “*” and N is the number of samples processed.

TFN: Transferrin; A1ATN: alpha 1 antitrypsin; PMGN: plasminogen; APO AIV P: apolipoprotein AIV precursor; HPTGN P: haptoglobin precursor; A1M/IATI P: alpha 1 microglobulin/ inter alpha trypsin inhibitor precursor; VTN: vitronectin; AK-1: adenylate kinase 1; GST A2: glutathione s-transferase A2; APO A-IV; apolipoprotein A IV; APO A-I: apolipoprotein A1.

In the Eβ thalassemic plasma samples transferrin (TFN), alpha 1 antitrypsin (A1ATN), plasminogen (PMGN), apolipoprotein A-IV precursor (APO A-IV P), haptoglobin precursor (HPTGN P), alpha 1 microglobulin/ inter alpha trypsin inhibitor precursor (A1M/IATI), Vitronectin and apolipoprotein A-I (APO A-I) shows a decrease in protein levels whereas adenylate kinase 1 (AK-1), apolipoprotein A-I (APO A-I) and glutathione s-transferase A2 (GST A2) show an increase in protein level compared to normal. Proteins such as TFN, AK-1 and apo A IV shows a very different trend compared to EE homozygous as well as high HbF conditions thereby indicating the specificity of the changes observed (Figure 2).

As most of the de-regulated proteins participate in multiple physiological processes like proteolysis, cargo-transport and iron homeostasis, their de-regulation might enlighten clinical manifestation. Eβ thalassemic patients irrespective of transfusion requirement, are exposed to oxidative stress and hypercoagulable state. Therefore we see decrease in the level of proteins such as HPTGN, TFN, APO A-I, A1M/IATI, VTN and PMGN. Those who have chronic anemia and are transfusion dependent undergo severe hemolysis, iron overload, hypercoagulability, thrombolytic events. Therefore the changes so observed in proteins such as HPTGN and AK-1, changes according to the severity and transfusion dependency of the patients (14). These 11 proteins if studied in greater details could be used collectively to monitor the condition of a patient and determining the effect of transfusion.
2. Urinary Proteomics of Eβ Thalassemia

In an attempt to establish a noninvasive method of disease monitoring we shifted our paradigm to urine proteomics. When we compared the urinary proteome of Eβ thalassemic samples compared to normal samples we found proteins such as A1ATN and APO A IV which shows a significant decrease in case of Eβ thalassemic samples similar to what we have seen in case of plasma. So far, proteins such as A1BG, A1ATN and TTN A show significant decrease in levels in case Eβ thalassemia. Interestingly proteins such as albumin (ALB), TFN and PMGN which are present in considerable amount in normal urine 2D gels were not detected in the 2D gels of Eβ thalassemic samples. A plausible explanation for this might be due to the fact that Eβ thalassemic patients utilises the TFN and PMGN to combat severe iron overload as well as hypercoagulability.

3. Lipidomics of Eβ Thalassemia

A comprehensive characterization of the major abundant lipid classes was performed leading to the identification of around 260 lipids in total distributed among 19 lipid classes in the plasma, erythrocytes and erythrocyte membrane fractions on combining the lipids identified in the positive as well as the negative ion mode.

In the plasma fractions of normal individual, lipids such as triacylglycerides (TAGs), cholesteryl esters (chol esters), lysophosphatidylcholines (LPCs) and phosphatidylcholines (PCs) are the most intense. Greater amount of LPCs and TAGs species were detected in the plasma fractions as compared to the other two fractions. Cholesteryl esters and diacylglycerides (DAGs) were only detected in the plasma fractions. Whereas, in case of erythrocytes and erythrocyte membrane fractions, lipids like PCs, sphingomyelin (SMs) and lysophosphatidylethanolamine (LPEs) are the most intense. Phosphatidic acids were observed only in case of erythrocytes and erythrocyte membrane. On comparing the lipidome of the Eβ thalassemic patients to that of normal, we have observed an increase in the ceramides
(Cer), and ethers of phosphatidylcholine (PC-O) populations in all three fractions. Lipids such as LPCs and LPEs showed a decrease in erythrocyte and erythrocyte membrane fractions whereas an increase in the plasma fraction. PCs and SMs show a decrease in all the fractions. The hydroxyl, keto, hydroperoxide, epoxy and polyhydroxy derivatives of long chain oxidized PCs were also detected in huge amounts in all three fractions, but a consistent increase in the Eβ thalassemic samples were observed only in case of erythrocyte and erythrocyte membrane fractions. The most abundant lipid species reported here also correlates with earlier studies\(^{(15)}\).

The changes observed clearly indicate that the erythrocytes are in a proapoptotic condition in the diseased samples. The premature eryptosis leading to acute anemia is one of the key pathological features of Eβ thalassemia. The changes in the lipidome combined with our already vast knowledge of the changes in the proteome in plasma as well as the erythrocytes may help us discover new insights so as to prolong the survival of the diseased erythrocytes. A detailed quantitative study of these changes might enable a “fingerprinting” approach towards a better understanding of the disease. This area has a huge potential in the therapeutic level as well as diagnostic level of this disease.

- **Urinary Proteomics of Urothelial Neoplasm**

  Albumin (ALB), Alpha 1 antitrypsin, Apolipoprotein A1, haemoglobin β subunit (Hbβ) and Transthyretin show an increase in expression in the urothelial neoplasm samples irrespective of their grade. Whereas proteins such as Transferrin show a greater increase in the high grade samples and Haptoglobin shows an increase in the low grade neoplasm samples. On the other hand, Inter alpha trypsin inhibitor heavy chain precursors show a decrease in both the grades. In certain cases, such as TTN and HP, the age matched control samples show an opposite trend suggesting that the changes observed in the neoplasm samples has no relevance to aging or medications.
CONCLUSION

From our proteomic study of two different body fluids (plasma and urine) we can see that proteins such as transferrin, plasminogen, apolipoprotein A-IV and alpha 1 antitrypsin play a significant role in maintaining homeostasis. The differential regulations of these proteins are suggestive of the stress the patient is undergoing. This study gives a set of proteins which can be further studied to help establish a robust disease and transfusion management system. Urine samples, which are easily obtainable, will help in a frequent and regular sample collection for monitoring disease conditions in a cost effective way.

Our lipidomics study shows the role that the lipids might play in the disease. The changes observed clearly indicate that the erythrocytes are in a proapoptotic condition in the diseased samples. The premature eryptosis leading to acute anemia is one of the key pathological features of Eβ thalassemia. The changes in the lipidome combined with our previous knowledge of the changes observed in the plasma proteome as well as the erythrocytes may help us discover new insights so as to prolong the survival of the diseased erythrocytes. A detailed quantitative study of these changes might enable a “fingerprinting” approach towards a better understanding of the disease. This area has a huge potential in the therapeutic level as well as diagnostic level of this disease.

HbEβ-thalassaemia because of changing phenotypes and variable medical interventions, it is difficult to accurately characterize the severity of this disease. Its remarkable phenotypic diversity is still not well understood\(^{(16)}\). We believe our study augments the knowledge already available.

REFERENCES


