CHAPTER 4

Comparison of membrane and hemoglobin-depleted cytosol proteomes of erythrocytes from normal, B-ALL and hereditary spherocytosis blood
4.1. Introduction

Like most of the other leukemic subtypes, B-ALL malignancies are presented with mild to severe anaemia. Several studies have reported abnormalities of the membrane cytoskeleton and enzyme activities of erythrocytes in myeloid as well as lymphoid leukaemias [143-147]. Although myeloid cells and erythrocytes arise from a common progenitor population, the lymphoid cells originate from lymphoid progenitors that are distinct from the myeloid progenitors. Hence a comparative study of the membrane and cytosol proteome of erythrocytes purified from peripheral blood of normal volunteers, B-ALL patients and patients suffering from a non-malignant haematological disorder like hereditary spherocytosis may add insights to our understanding of altered erythrocyte physiology in leukaemia vs. erythroid disorders.

Hereditary Spherocytosis (HS) is a common inherited membranopathy characterized by phenotypic and genotypic heterogeneity. It occurs in all racial groups but, is more common in North-European and Japanese populations [148]. HS is commonly associated with dominant inheritance although non-dominant and recessive inheritance is also known. Spheroidal red cells in HS are characterized by decreased deformability and a reduced surface to volume ratio. Several mutations of α- and β-spectrin, ankyrin, band 3 and band 4.2 are known to be associated with HS. The consequence of the primary mutations is loss of vertical linkage between membrane skeleton and lipid bilayer leading to membrane loss and decrease in membrane surface area, compromising normal erythrocyte deformability in circulation [149].

Previous work from our laboratory showed higher loss of transbilayer phospholipid asymmetry in HS erythrocytes compared to normal [150]. Clinical severity of HS ranges from asymptomatic condition to life-threatening anemia with transfusion-dependence and rarely to hydrops fetalis and fetal death. The same protein deficiency may account for a mild or severe disease phenotype. This heterogeneity appears to be related to the coinheritance of modifying genes and secondary protein deficiencies triggered by the primary defect [151-153].
Profiling of erythrocyte proteins has recently gained importance and erythrocyte disorders like sickle cell anemia have been addressed by the proteomic approach [154, 155]. Proteomic analysis of erythrocyte cytosol had been handicapped by the large abundance of hemoglobin, masking majority of the other cytosolic proteins. But in last couple of years, a few novel hemoglobin depletion methods and highly sensitive mass spectrometry have emerged [116, 156-158].

We have found significant changes in B-ALL erythrocyte cytosol and membrane proteomes that may, in part, be responsible for the observed anaemia and may add new insights in understanding the heterogeneous presentation of the disease.

4.2. Materials and Methods

4.2.1. Materials
Sulphopropyl sephadex (SP sephadex), percoll, CHCA (α-cyano-4-hydroxycinnamic acid) MALDI matrix were obtained from Sigma (St. Louis, MO). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). Sequencing grade trypsin was obtained from Promega (Madison, WI). Other in-gel tryptic digestion reagents were obtained from Pierce Biotechnologies (Bedford, MA). All other reagents, if not mentioned otherwise, were purchased locally and were of proteomics grade.

4.2.2. Sample Collection
Peripheral blood samples of healthy normal volunteers, B-ALL patients and HS patients, diagnosed for the first time at Department of Hematology, Ramakrishna Mission Seva Pratisthan, Kolkata, India, were collected, with respective consents (parents in case of minors). The institutional ethical review board approved the whole experimental procedure. Red blood cells were purified to >99.5% purity using 75% percoll, as described earlier [116, 123].

4.2.3. Hemoglobin depletion
Hemoglobin depletion was carried out as described earlier [116, 123]. In brief, hemoglobin A
and A2 were kept bound on the SP-sephadex matrix at pH 6.7 while other proteins were obtained in the flow-through.

4.2.4. 2DGE of erythrocyte cytosol

2DGE of hemoglobin-depleted red blood cell cytosol, and staining with CBB or SYPRO-RUBY were essentially the same as described earlier [116, 123]. Densitometry analysis of the gel spots of interest was performed using the density tools of PDQuest (V 7.1) software package. Spot volume (intensity) of the desired spot(s) was normalized as parts per thousand of the total spot volume using the spots that were present in all gels, to find out the relative abundance of a spot in a sample.

4.2.5. 2DGE of Erythrocyte ghost

The erythrocyte ghost membranes were prepared following protocols described earlier [159] with minor modifications. In brief, erythrocytes were lysed in 5mM phosphate buffer, pH 7.4, containing 1mM EDTA and 0.1mM PMSF, overnight at 4°C to obtain erythrocyte ghost membranes. Membranes were washed 5 times with the lysis buffer, pelleted by centrifugation at 30,000g and dissolved in 2D sample buffer containing 20mM Tris, 5M urea, 2M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10 and 0.2%(w/v) Biolyte 3-10 ampholyte (Bio-Rad) and Protease inhibitor (Roche Diagnostics, Germany). Just prior to IPG strip rehydration, erythrocyte membrane proteins were reduced with 1% (v/v) TBP (Bio-Rad) for 60 minutes at room temperature, and alkylated with 20 mM IAA in dark for 90 minutes at room temperature. 800μg of erythrocyte membrane proteins were focused in 3-10 NL IPG strips (Bio-Rad) up to 1, 20, 000 Volt Hours, followed by SDS-PAGE in 8-16% gradient gels. Resulting 2D gels were silver stained following established protocol. For MS-based identification, SYPRO-RUBY stained gel spots (Bio-Rad) were used. Densitometry analysis of the gel spots of interest was the same as done for erythrocyte cytosol 2D gels.

4.2.6. MALDI TOF/TOF mass spectrometry

The protein spots from CBB and SYPRO RUBY-stained 2D gels were digested with trypsin
according to Shevchenko et al. [36] with minor modifications using Trypsin Gold from Promega (Madison, WI, USA). MS of the digested peptides was carried out in a MALDI-TOF/TOF tandem mass spectrometer (Applied Biosystems, AB 4700). Recrystallized CHCA and 2, 5-dihydroxy-benzoic acid (Sigma) were used as matrices. PMFs were acquired in positive reflector mode. Autotrypsin and common keratin peaks were first validated and subsequently excluded from MS/MS analysis. Ten most intense peptides from CBB-stained gels and seven most intense peptides from SYPRO RUBY-stained gels were subjected to MS/MS analysis. Peak lists were prepared from raw MS and MS/MS data using GPS explorer V3.0 (Applied Biosystems) software and noise reduction and de-isotoping were performed using default settings. The resulting PMF and MS/MS data were searched against human MSDB and Swiss-Prot databases using in-house MASCOT version 2.0.05 (Matrix Science, UK) server and the MOWSE score was considered to determine hits. Proteins with at least two MS/MS hits with p<0.05 were considered a ‘true hit’. Also the hit for the next best non-homologous protein was accepted and only the proteins with considerable difference from a nonspecific protein hit were taken into account. For different homologous proteins having the similar MOWSE scores, preference was given to the protein with best match between theoretical and experimental molecular weight and pI. All MS experiments were repeated at least thrice. The database search parameters included one missed cleavage, error tolerance of ± 100 ppm for PMF and ± 1.2 Da for MS/MS ion search. Some common variable modifications like carbamidomethyl cysteine, methionine oxidation, and N-terminal acetylation were included. All MS/MS peaks matched by this method were manually checked and confirmed.

4.2.7. Statistical Analysis

Densitometry data from erythrocyte ghost and cytosol 2D gels were subjected to unpaired two-tail Student’s t-test for evaluating significance of the differences observed between normal, B-ALL and HS individuals.
4.2.8. Transmission Electron Microscopy of erythrocyte ghost preparations

Erythrocyte membrane preparations were laid on formver/carbon-coated copper grids of 600 meshes and stained with 0.5% (w/v) phosphotungstic acid with a staining time of 25-30 seconds. The grids were dried properly before viewing under the Tecnai S Twin, FEI electron microscope (Eindhoven, The Netherlands) operating at 200 kV accelerating voltage elaborated earlier [160].

4.3. Results

4.3.1. Differences in the erythrocyte cytosol proteomes of B-ALL, HS and normal samples

B-ALL and HS patients, who had never received blood transfusion, were used for erythrocyte cytosol proteome analysis. Representative hemoglobin-depleted erythrocyte cytosol proteomes from a B-ALL patient, an HS patient and a normal control have been shown in Figure 4.1.

*Figure 4.1:* Representative 2DGE maps of hemoglobin-depleted erythrocyte cytosol from normal, B-ALL and HS individuals. 1- peroxiredoxin 2; 2- thioredoxin; 3- superoxide dismutase; 4- nucleoside di-phosphate kinase; 5- suppressor of tumorigenicity 13; 6- aldehyde dehydrogenase; 7- heat shock protein 70.

The mass spectrometry details of the proteins are given in Table 4.1. The 2D gels of B-ALL and HS cytosol were markedly different from that of normal controls. While HS erythrocyte
cytosol showed up-regulation of redox regulators and down-regulation of a co-chaperone ST13 and a nucleotide kinase NDPK (adjoining histogram plot); the hemoglobin-depleted erythrocyte cytosol proteome of B-ALL patients showed somewhat opposite trends with down-regulation of an oxidoreductase (aldehyde dehydrogenase) and pI shift of a chaperone (HSP-70).

Table 4.1: Identification of differentially regulated proteins by MALDI TOF/TOF mass spectrometry in Hemoglobin-depleted-erythrocyte cytosol proteome

<table>
<thead>
<tr>
<th>Name of the Protein</th>
<th>Accession ID</th>
<th>MW / pI</th>
<th>Mascot Score</th>
<th>Sequence Coverage</th>
<th>MS/MS peaks matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxiredoxin 2 (PRDX2)</td>
<td>PRDX2_HUMAN</td>
<td>21.7 / 5.67</td>
<td>216</td>
<td>62%</td>
<td>4</td>
</tr>
<tr>
<td>Thioredoxin (TRDX)</td>
<td>P10599</td>
<td>11.6 / 4.8</td>
<td>309</td>
<td>90%</td>
<td>5</td>
</tr>
<tr>
<td>Superoxide Dismutase (SOD)</td>
<td>P00441</td>
<td>15.9 / 5.9</td>
<td>465</td>
<td>63%</td>
<td>4</td>
</tr>
<tr>
<td>Nucleoside Di-phosphate Kinase (NDPK)</td>
<td>1NSKR</td>
<td>17.3 / 7.0</td>
<td>144</td>
<td>56%</td>
<td>2</td>
</tr>
<tr>
<td>Suppression of Tumorigenicity 13 (ST13)</td>
<td>P50502</td>
<td>41.3 / 5.2</td>
<td>206</td>
<td>33%</td>
<td>6</td>
</tr>
<tr>
<td>Aldehyde Dehydrogenase 1 (ALDH)</td>
<td>P00352</td>
<td>54.8 / 6.3</td>
<td>147</td>
<td>47%</td>
<td>6</td>
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</table>

4.3.2. Differences in erythrocyte membrane proteomes of B-ALL, HS and normal samples

B-ALL and HS patients, who had never received blood transfusion, were also used for the erythrocyte ghost proteome analysis. Representative erythrocyte membrane proteomes from a B-ALL patient, an HS patient and a normal control have been shown in Figure 4.2.
Figure 4.2: Representative normal, B-ALL and HS erythrocyte membrane proteomes. 1-Hemoglobin; 2- Band 4.1; 3- Ankyrin; 4- Tropomyosin 3; 5- Tropomyosin 1; 6- Calpastatine; 7- Dematin; 8- Spectrin; 9- Glyceraldehyde 3 phosphate dehydrogenase (G3PD); 9A- G3PD fragments

The mass spectrometry details of the proteins are given in Table 4.2.

Table 4.2: Identification of differentially regulated proteins by MALDI TOF/TOF mass spectrometry in erythrocyte membrane proteome

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession ID</th>
<th>MW / pI</th>
<th>Mascot Score</th>
<th>Sequence coverage</th>
<th>MS/MS peaks matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>HBBWB</td>
<td>16.0/7.3</td>
<td>99</td>
<td>78%</td>
<td>4</td>
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<tr>
<td>Band 4.1</td>
<td>MMHUE4</td>
<td>95.2/5.37</td>
<td>174</td>
<td>32%</td>
<td>5</td>
</tr>
<tr>
<td>Ankyrin</td>
<td>SJIHUK</td>
<td>20.6/5.65</td>
<td>183</td>
<td>26%</td>
<td>7</td>
</tr>
<tr>
<td>Tropomyosin 3</td>
<td>Q5VUS8_HUMAN</td>
<td>28.9/4.72</td>
<td>241</td>
<td>62%</td>
<td>10</td>
</tr>
<tr>
<td>Tropomyosin 1</td>
<td>Q1ZYL5_HUMAN</td>
<td>28.5/4.75</td>
<td>138</td>
<td>62%</td>
<td>9</td>
</tr>
<tr>
<td>Calpastatine</td>
<td>CAA03059</td>
<td>74.9/4.99</td>
<td>86</td>
<td>36%</td>
<td>2</td>
</tr>
<tr>
<td>Dematin</td>
<td>DEMA_HUMAN</td>
<td>45.5/8.9</td>
<td>85</td>
<td>42%</td>
<td>4</td>
</tr>
<tr>
<td>Spectrin</td>
<td>Q59FP5_HUMAN</td>
<td>246/5.1</td>
<td>275</td>
<td>30%</td>
<td>12</td>
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<tr>
<td>G3PD</td>
<td>DEHUG3</td>
<td>36.0/8.6</td>
<td>151</td>
<td>60%</td>
<td>2</td>
</tr>
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</table>
Due to the inherent complexity of a 2D gel-based membrane proteomic studies, we have only concentrated on the spots which were very significantly different (p<0.001) between normal, B-ALL and HS membrane proteomes. Comparison of the erythrocyte membrane proteomes on 2DGE led to the observation of elevated levels of membrane associated globin chains and reduced membrane association of glyceraldehydes-3-phosphate dehydrogenase (G3PD) in both B-ALL and HS patients. While increased association of globin chains with the spherocyte membranes in HS directly indicate severe oxidative stress in the spherocytes, membrane-bound denatured/oxidized hemoglobin and defective erythrocyte cytoskeletal network in B-ALL may be implicated in the erythrocyte destruction in spleen, making the patients anaemic. The HS erythrocyte membrane proteome also displayed increased levels of low molecular weight fragments of several major cytoskeletal proteins (adjoining histogram plot).

4.3.3. Alterations in HS erythrocyte ghost morphology

Transmission electron microscopy (TEM) of the sealed erythrocyte ghosts showed significant changes in the ultra-structure of B-ALL and HS erythrocyte membranes with an impression of disrupted membrane skeletal architecture, as shown in Figure 4.3.
4.4. Discussion

This proteomic study reveals differential expression of major cytosolic redox regulator proteins in the erythrocyte cytosol from B-ALL and HS samples. Mice expressing low levels of redox enzymes die within three days under high oxidative stress. The lifespan of their erythrocytes under normal conditions is half that of the wild type, suggesting that reactive oxygen species regulate erythrocyte lifespan [161]. SOD deficiency causes anemia in mice [162] and TRDX is up-regulated in sickle cell anemia on hydroxyurea treatment [163]. The elevated level of PRDX2 in HS hints towards its high demand to scavenge elevated levels of reactive oxygen species in the spherocyte. The concomitant up-regulation of two other redox regulators, TRDX and SOD, indicates an adaptive response of spherocytes to oxidative damage. We have previously reported up-regulation of PRDX2, TRDX and SOD also in HbEβ-thalassemic erythrocyte cytosol [123]; and hence speculate that the pathophysiology of the two erythrocyte disorders may be significantly related. Aldehyde dehydrogenase (ALDH) constitutes a group of oxidoreductases that catalyse oxidation of aldehydes and include the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase (G3PD). About 90% of erythrocyte G3PD is bound to membrane in its inactive form, becoming active when released into the cytoplasm [153]. B-ALL erythrocytes showed reduced levels of membrane associated G3PD and cytosolic
ALDH. Deficiency of G3PD has also been described in case of CML [147]. Altered enzyme activity observed in B-ALL may either be due to intracellular stress with temporary inhibition of red cell glycolysis, posttranslational molecular modification or cyto and karyokinetic abnormalities with loss of functional genetic material governing enzyme synthesis, leading to enzyme abnormality. The red cell enzyme changes may reflect production of an abnormal clone of red blood cell and that these may arise from altered stem cells, has also been suggested by some studies [147]. The defective enzyme activities may be the result of derivation of abnormal cell lines form an abnormal pluripotent stem cell. The presence of such a pluripotent cell has been demonstrated by the presence of a Ph chromosome not only in myeloid precursors but also in erythroid precursors in CML [147].

The observed reduction in membrane associated G3PD and concomitant rise in ALDH amounts in the spherocyte cytosol might reveal a mechanism of erythrocyte metabolic regulation in times of crisis like oxidative stress. The oxidative stress induced time dependent loss of membrane bound G3PD in intact red cells [164] supports the hypothesis.

Membrane bound haemoglobin (MBH) is used as oxidative stress marker [165]. Increased association of globin chains with the erythrocyte membranes in B-ALL and HS emphasize that erythrocytes are subjected to severe oxidative stress in both the haematological disorders. Membrane-bound denatured/oxidized hemoglobin has also been implicated in the proposed erythrocyte clearance mechanism, through the induction of band 3 clustering on the membrane, which in turn leads to autologous IgGs and complement binding and consequent erythro-phagocytosis [152].

We have also investigated the electron micrograph and membrane proteome of erythrocytes in cases of severe non-HS hemolytic anemia [166], and the profile highly matches with that observed in B-ALL and HS patients. The extent of deregulations in hemolytic anemia is somewhat higher than that in B-ALL or HS. Figure 4.4 shows the 2DGE profile of erythrocyte membrane in a case of severe non-HS hemolytic anemia.
In conclusion, the present work is the first report of 2DGE based proteomic investigation of B-ALL and HS erythrocyte cytosol, after hemoglobin depletion along with the membrane, and describes a spectrum of secondary protein alterations that accompany the primary defects. Secondary protein deficiencies are often observed and may have serious implications in the clinical outcome of the disease. Changes in the erythrocyte proteomes directly indicate altered redox-regulation, metabolism, protein degradation, cytoskeletal disorganization and oxidative stress. The ultra-structural changes observed in the B-ALL and HS erythrocyte membranes also indicate drastic changes in the architecture of the spectrin-based membrane skeleton. Previous reports support our findings [144]. The aforementioned abnormalities in skeletal, integral or anchoring proteins results in the inability of the erythrocyte to control its surface area or cell volume and causes shortening of erythrocyte survival. Although the examined group of affected individuals is rather small, it is of probable clinical importance that the aberrant electrophoretic profiles were detected in all patients and were similar to that observed in non-HS hemolytic anemia patients. We propose our data to add some new insights in the field of B-ALL pathophysiology, clinical heterogeneity and disease
progression. Since the treatment strategies in B-ALL must address the whole spectrum of the pathophysiology of the disease, much is expected from future studies on erythrocyte proteomes, signaling mechanisms and oxidatively damaged components in B-ALL erythrocyte membrane.