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Quantitative analysis of the erythrocyte membrane proteins in polycythemia vera patients treated with hydroxycarbamide

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ABSTRACT

More than 90% of polycythemia vera (PV) patients have a mutation in the protein JAK2, which is closely associated with the erythrocyte membrane. With the comparison of 1-D gels of erythrocyte membranes obtained from PV patients treated with hydroxycarbamide and those of untreated controls we observed significant differences in the region of 40–55 kDa. On the basis of the LC–MS/MS analysis of this region we report up-regulation of four protein disulfide isomerases, which was subsequently confirmed by targeted mass spectrometric analysis. In further studies it will be prudent to compare this in patients both treated and not treated with hydroxycarbamide.

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1. Introduction

Polycythemia vera (PV) is a myeloproliferative neoplasm originating from the clonal proliferation of a pluripotent hemopoietic progenitor characterized by erythrocytosis, which is an increase in the red blood cell mass. Erythrocytosis, which is the hallmark of PV, can result in life threatening clinical effects such as venous or arterial thrombosis [1]. In 2005, a unique acquired mutation was identified in the cytoplasmic tyrosine kinase JAK2 (Janus kinase 2) in myeloid cells of over 90% of PV patients. It has since been demonstrated that the JAK2 V617F mutation plays a central role in the diagnosis of PV and can be used as a way of differentiating neoplastic myeloid proliferations from reactive ones [1,2]. The V617F mutation is known to result in the production of a constitutively activated JAK2 protein, which autophosphorylates JAK2 itself [3]. This in turn leads to cytokine independent erythrocytosis [4]. EpoR is a transmembrane receptor, which, in a healthy person, requires binding of its ligand, Epo, to its extracellular domain. The binding initiates the process of phosphorylation by JAK2, which is associated with the cytoplasmic domain of EpoR in close proximity to the erythrocyte membrane [5,6].

Mass spectrometry based proteomics technologies have successfully been used to establish protein profiles of the mature erythrocyte, including post-translational modifications [7–11]. In contrast to proteomics, the absence of a nucleus in the mature erythrocyte has made mRNA based micro array approaches inapplicable. As no convenient biomarker exists today for PV, screening the erythrocyte content by differential proteomics could help in finding more efficient diagnosis tests [12]. Alterations of erythrocyte membrane associated proteins (ERMBPs) have caused erythrocyte membrane disorders such as hereditary spherocytosis and hereditary elliptocytosis. Therefore ERMBPs have been the focus of investigation for several hematological disorders in humans [13–15]. As erythrocytosis is a characteristic feature of PV disease and JAK2 protein is closely associated with the erythrocyte membrane, protein abundance changes in the erythrocyte membrane is an ideal target to study mechanisms of this disorder. Although the defect arises from bone marrow stem cells of PV, pathological changes may occur in the mature erythrocyte. For instance, in chronic myeloid leukemia which is one of the myeloproliferative disorders like PV, patients suffer from anemia, and neutrophilic cathepsin G from serum mediates cleavage of erythrocyte membrane protein band 3, which enhances phagocytosis of erythrocytes [16]. In previous studies of PV surface-enhanced laser desorption/ionization mass spectrometry has been used for the analysis of serum proteins, differentiating two PV sub groups and also PV from essential thrombocythemia [17,18]. As a target for erythrocyte proteomics, analysis of erythrocyte membrane fractions would also be interesting as it sets the stage for protein expression and function-based activity profiling [7,8,10].

In this paper, we will focus on the quantitative proteomics analysis of erythrocyte membranes in PV patients with the JAK2 V617F mutation and healthy controls. At the time of investigation all patients were treated with hydroxycarbamide, which is a metabolic inhibitor of ribonucleoside reductase and a non-alkylating myelosuppressive agent used to control the erythrocytosis [19]. Using 1-D gel separation and a liquid chromatography–tandem mass spectrometry (LC–MS/MS) based label-free quantitative proteomics approach, we found four potential differentially regulated ERMBPs. The observed increased abundance of these candidates was validated by selected reaction monitoring (SRM) [20–22]. The four proteins belong to the protein disulfide isomerase family (PDI), which is involved in oxidative stress response [23]. In keeping with this, increased oxidative stress has been previously reported in the serum of PV patients, even though treatment alleviates the stress in part [24]. In future studies, further validation of these candidate markers are planned to evaluate their diagnostic potential for the detection of PV.

2. Materials and methods

2.1. *Patients and controls*

Blood samples were obtained from five PV patients with the JAK2 V617F mutation (P1–P5) and five healthy volunteers (N1–N5). All 5 patients were treated with hydroxycarbamide and a low aspirin dose for over 6 months, displayed stable blood counts (Table 1) and had also been subjected to multiple therapeutic venesections. The patients (4 males and 1 female) were between 40 and 60 years of age, while the healthy controls (2 males and 3 females) were between 40 and 50 years of age. A full blood count (FBC) was performed in all patients with a Beckman Coulter hematology analyzer. Ethical clearance was obtained from the Ethical Review Committee of Faculty of Medicine, University of Colombo (Reference No: EC-11-007).

2.2. *Purification of erythrocyte membrane proteins*

Each of the 10 blood samples was mixed with 4%, w/v, EDTA, pH 7.4 as an anticoagulant at a ratio of 1:20. All the blood samples were kept 4 days at +4 °C before analysis in order to mature reticulocytes to erythrocytes. Erythrocytes were obtained from the whole blood by washing it four times with phosphate buffered saline, pH 8.0 employing low speed centrifugation, 1500 × *g* for 10 min. A pellet of the washed erythrocytes was kept at −80 °C for further analysis. Erythrocyte membrane fractions were isolated by osmotic lysis of the washed erythrocytes using lysis solution (5 mM phosphate buffer, pH 8.0, 1 mM EDTA) followed by high speed centrifugation, 24,000 x g for 10 min [7]. This was repeated for 3 more times and hemoglobin was removed with the supernatant in each time. Protease inhibitors (Complete mini EDTA-free, Roche Diagnostics GmBH, Mannheim, Germany) were added to the lysing solution as well as to the washing solutions. Finally, the isolated membranes were solubilized by 4% Triton X-100 in 10 mM Tris–HCl, pH 8.8. Total protein amounts were estimated by the Lowry method (DC Protein assay, Bio-Rad laboratories, Richmond, USA) using BSA for the standard protein and also by measuring absorbance at A280 nm with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Table 1 – Full blood count parameters and Jak2 status of PV patients (P1–P5) and controls (N1–N5).

	PV patients					Healthy controls					Ref. value
	P1	P2	P3	P4	P5	N1	N2	N3	N4	N5	
JAK2 (V617F)	(+)	(+)	(+)	(+)	(+)	(–)	(–)	(–)	(–)	(–)	(+) or (–)
WBC ($\times 10^9$/l)	14.2	10.9	10.8	14.7	16.7	5.4	4.8	6.7	6.3	5.8	4.0–11.0
Hb (g/dl)	12.4	15.9	13.8	14.8	10.4	13.3	14.5	13.7	13.6	12.7	12–17
RBC Count ($\times 10^{12}$/l)	6.2	5.8	5.4	6.0	5.0	4.5	5.0	4.9	4.8	4.5	4.0–6.0
HCT (%)	39.7	49.4	43.2	47.3	34.7	39.7	43.2	41.4	40.7	37.8	35–50
Platelet count ($\times 10^9$/l)	388	140	345	332	155	280	272	297	257	307	150–450
MCV (fl)	64.1	85.0	79.6	78.7	68.4	87.8	85.8	85.2	85.1	84.9	78–95
MCH (pg)	20.0	27.4	25.4	24.6	20.5	29.4	28.8	28.2	28.4	28.5	27–32
MCHC (%)	31.2	32.2	31.9	31.3	30.0	33.5	33.5	33.1	33.4	33.6	30–35

MCHC, mean cell hemoglobin concentration.

2.3. *1-D SDS-PAGE and in-gel trypsin digestion of proteins*

Twenty micrograms of the erythrocyte membrane proteins of PV patients and healthy controls were separated on pre-cast Tris-acetate gels (Criterion XT, 3–8% acrylamide, Bio-Rad) using Tricine running buffer. Proteins were silver stained as described previously [25]. Gel bands of interest were cut, dehydrated with 100% acetonitrile and stored as dried ones at −20 °C. The proteins were digested in-gel with trypsin according to the original protocol of Shevchenko et al. [26] with minor modifications. Proteins were reduced with 20 mM DTT and alkylated with 55 mM iodoacetamide, prepared in 50 mM NH_4HCO_3. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/μl trypsin (Promega, Madison, USA) on ice. After 10 min, more 50 mM NH_4HCO_3 was added to the gel pieces to cover them, and digestion was allowed to proceed overnight at 37 °C. The peptides were extracted by 50% acetonitrile, 5% formic acid. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to MS analysis, the peptides were dissolved in 1% formic acid. Total peptide amounts were estimated by measuring absorbance at A_{280} nm with the NanoDrop 1000 spectrophotometer.

2.4. *In-solution trypsin digestion of proteins for SRM analysis*

The isolated membrane proteins from PV patients and healthy controls were subjected to in-solution digestion. The proteins were reconstituted with 6 M urea, reduced with 10 mM DTT and alkylated with 40 mM iodoacetamide prepared in 25 mM NH_4HCO_3. Before adding trypsin the solution was diluted 10 times with 25 mM NH_4HCO_3. The digestion was allowed to proceed overnight at 37 °C with the additon of typsin at ratio of 1–30 by weight. The Triton X-100, which was used for the membrane protein isolation, was removed with Pierce detergent removal spin columns (Thermo Fisher Scientific). Following acidification of the solution containing tryptic peptides with 5% formic acid, the peptides were desalted using Empore C18 columns (3M, St. Paul, USA), then dried by vacuum centrifugation. Prior to MS analysis, the peptides were dissolved in 1% formic acid. Total protein amounts were estimated with the NanoDrop 1000 spectrophotometer.

2.5. *LC–MS/MS for identification and non-targeted quantification of proteins*

The in-gel digests were analyzed using a nanoflow liquid chromatography system (Ultimate 3000, Dionex, Sunnyvale, USA) coupled to a QSTAR Elite ESI-hybrid Q-TOF tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada). Five microliters of peptide solutions (200 ng) were first loaded on a pre-column (0.3 mm × 5 mm, PepMap C18, 5 μm, 100 Å, LC Packings, Sunnyvale, USA) at a flow rate of 18 μl/min and subsequently separated on an in-house packed analytical column (75 μm × 15 cm, Magic C18AQ, 5 μm, 200 Å, Michrom BioResources, Sacramento, USA) at a flow rate of 200 nl/min. The mobile phase consisted of water/acetonitrile (98:2 (v/v)) with 0.2% formic acid (solvent A) or acetonitrile/water (95:5 (v/v)) with 0.2% formic acid (solvent B). LC gradient elution condition was 2–35% B (0–20 min), 35–95% B (20–25 min), 95% B (25–40 min), and then 2% B (40–45 min) with a flow rate of 200 nl/min. MS data was acquired automatically using Analyst QS 2.0 software (Applied Biosystems/MDS Sciex). An information dependent acquisition method consisted of a one second TOF MS survey scan with a mass range 350–1500 *m/z* and maximum two seconds product ion scans with a mass range 50–2000 *m/z*. Two most intensive precursor ion peaks, with ion counts over 20 and charge states 2+ or 3+, were selected for fragmentation. Dynamic exclusion duration was 60 s.

2.6. *LC–MS/MS for creating a spectral library*

For designing a targeted quantitative method for the validation of the nine ERMBPs targets, first the in-solution digests from one healthy control (N4) and one PV patient (P2) were analyzed using an Easy-nLC 1000 nanoflow liquid chromatography system coupled to a Q Exactive ESI-hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific). Five microliters of the peptides (500 ng) were first loaded on an in-house packed pre-column (100 μm × 2 cm, Magic C18AQ), and subsequently separated on an in-house packed analytical column at a flow rate of 300 nl/min. The analytical column and the mobile phases were the same as those used for the QSTAR Elite system. LC gradient elution condition was 2–35% B (0–40 min), 35–100% B (40–42 min), and then 100% B (42–50 min), with a flow rate of 300 nl/min. Data dependent acquisition was performed in positive ion mode. MS spectra were acquired from *m/z* 300 to *m/z* 2000 at a resolution of 70,000

at *m/z* 200 with a target value of 1,000,000 and maximum injection time of 120 ms. The 10 most abundant precursor ions of which charge states were 2+ or higher were selected for higher energy collisional dissociation with an isolation window of 2 and normalized collision energy of 27. MS/MS spectra were acquired at a resolution of 17,500 at *m/z* 200 with a target value of 20,000, maximum injection time of 250 ms, and the lowest mass fixed at *m/z* 100. Dynamic exclusion duration was 10 s.

2.7. LC–MS/MS for SRM

A TSQ Vantage ESI-triple quadrupole mass spectrometer (Thermo Fisher Scientific) was used for SRM analysis of the in-solution digests. The LC system and conditions of chromatographic separation were same as described for the Q Exactive system. Five hundred nanograms of the in-solution digests were injected on the pre-column coupled to the analytical column for separation. The mass spectrometer was controlled by Xcalibur software (version 2.2, Thermo Fisher Scientific) and operated in the positive ion mode. A spray voltage of 2.3 kV, an ion source temperature of 275 °C, and Q1 and Q3 set to unit/unit resolution (0.7 Da), 0.002 *m/z* scan width, and a dwell time of at least 50 ms was used. Using the optimized method (described below), the in-solution digests of PV patients and healthy controls were analyzed in duplicate. All the Raw files were imported to Pinpoint 1.2 software (Thermo Fisher Scientific) and data analysis was performed.

2.8. Database searches and gene ontology annotations

Database searches of the QSTAR Elite data were performed by Mascot (version 2.2) (Matrix Science, London, UK) search engine against Swiss-Prot database (2011_08) using a *Homo sapiens* taxonomy filter (20,245 sequences). Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionines was set as a variable modification. One missed trypsin cleavage site was allowed. Mass tolerance for MS and MS/MS was set to 0.3 Da, and only proteins identified with minimum two unique peptides were selected. In case of the Q Exactive data, Mascot search was preformed via Proteome Discoverer (version 1.3, Thermo Fisher Scientific) with the same parameters but mass tolerances for MS and MS/MS were set to 10 ppm and 0.02 Da, respectively. Scaffold software (version 3.3.2, Proteome software) and Uniprot database were used to provide Gene Ontology (GO) annotations to proteins.

2.9. Non-targeted label-free protein quantification

Relative quantification of the in-gel digested proteins was carried out by both spectral counting and by measuring precursor ion intensity. Peptide-spectrum matches obtained from Mascot results were counted manually for the identified proteins, using unique peptides (required bold) with an expectation value <0.05. The spectral counts were normalized to housekeeping proteins, actin or basigin. For the proteins elected for further quantification, the associated unique peptides were monitored with the extracted ion chromatograms (XICs, ±0.5 *m/z*), and the intensities of all the peptide ions were summed to assess the protein abundances [12]. Relative abundance of the protein was obtained after normalizing to basigin.

2.10. SRM method development

The preliminary analysis of in-solution digests on the Q Exactive resulted in the identification of 193 proteins (Scaffold, 99% protein confidence, 95% peptide confidence, minimum 2 peptides per protein), of which 135 were annotated as membrane proteins. This data covered all the proteins targeted in SRM (see the list of proteins below). Based on the Q Exactive data and the QSTAR Elite data (in-gel digests), proteotypic peptides were chosen as surrogates of the measured proteins according to the following rules: zero missed cleavages, carbamidomethylation as a fixed modification, no methionine residues present, excluded ragged ends and known post-translational modifications. Up to six transitions per peptide precursor ion were chosen, and up to six peptides (minimum 3) per protein were included in the list. Transitions for proteotypic peptides were selected based on spectral libraries created from the former data dependent experiments with the Q Exactive and QSTAR Elite, when no match was found the PeptideAtlas was addressed.

To optimize the method, one of the PV patient samples (P1 in-solution digest) was used. Retention times were predicted based on the spectral library built from the Q Exactive data to create a preliminary scheduled SRM method for optimization. The iSRM technique (Thermo Fisher Scientific) was applied in order to confirm the identity of each peptide and to choose the most intense transitions for quantitative analysis. The peptides were targeted in a 3-min scheduled acquisition window, monitoring for 2 primary and 4 secondary transitions for each peptide. Once the intensity of the two transitions overcame the threshold of 300, instrument triggered the intensity of six additional transitions for the same peptide. The acquired MS/MS spectra were then compared with the library spectra to confirm that the major MS/MS peaks matched. For quantitative runs the four most intense transitions per peptide were monitored within a 3 min time window. The final list of targets included; 10 proteins, 36 peptides, 113 transitions (Supplementary Table 1). The collision energy (CE) predicted by Pinpoint software (according to the equation: $CE = 0.04\,m/z - 3.88$, for doubly; and $CE = 0.04\,m/z - 0.89$, for triply charged ions), was optimized for each transition in 5 steps of 3 eV. The best CE value for each transition was chosen automatically in Pinpoint software.

For each of four transitions the area under the curve was calculated by Pinpoint program separately, and these values were then summed up to give the final peptide abundance in the sample. These values were used to calculate protein abundance, which was then normalized by total area of the reference protein basigin. Results were shown as relative abundance ratios of proteins, patients to controls.

2.11. Statistical analysis of differentially regulated proteins

XIC and SRM data were normalized to the house-keeping protein basigin before statistical data analysis. The normalized data obtained from PV patients and healthy controls were subjected to nonparametric Wilcoxon rank sum test (Mann–Whitney test) using SPSS (Statistical Product and Service Solutions) software (version 20).

3. Results and discussion

3.1. *Hematological parameters*

The FBC results of patients and controls are summarized in Table 1. The white blood cell count (WBC) of the patients (P1–P5) ranged from 10.8 to 16.7 $\times 10^9$/l with a mean value of 13.5 $\times 10^9$/l. This was considerably higher than the WBC of the control group (N1–N5) which ranged from 4.8 to 6.7 $\times 10^9$/l with a mean value of 5.8 $\times 10^9$/l. This is in accordance with a characteristic feature of PV as it results in a panmyelosis with patients frequently having a leucocytosis. The red blood cell count (RBC) of the patients ranged from 5.0 to 6.2 $\times 10^{12}$/l with a mean value of 5.7 $\times 10^{12}$/l and was higher than the control group, which had a RBC ranging from 4.5 to 5.0 $\times 10^{12}$/l with a mean value of 4.7 $\times 10^{12}$/l. The hemoglobin level in four patients and all five controls were within the normal range. One patient (P5) was anemic (hemoglobin (Hb) = 10.4 g/dl) with a low mean cell volume (MCV) and mean cell hemoglobin (MCH) which was likely to be due to iron deficiency following repeated venesections. The hematocrit (HCT) of all five patients and controls was within the normal range. Although the HCT of patients was well controlled by venesections in combination with hydroxycarbamide, these treatments were not aiming at altering the underlying pathogenesis of the disease. The platelet count of all but one patient (P2) was within the normal range. The marginally low platelet count of 140 $\times 10^9$/l in P2 is likely to be the effect of hydroxycarbamide. The patients did not have other secondary complications. Peripheral blood film examination did not reveal features suggestive of myelofibrosis or excess blasts. The reticulocyte

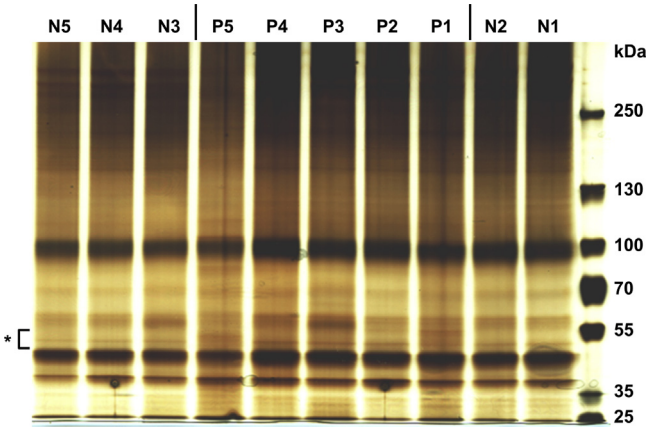

Fig. 1 – ERMBPs of PV patients (P1–P5) and healthy controls (N1–N5) separated by SDS-PAGE. A representative gel is shown (silver staining). An asterisk indicates a gel area of interest (40–55 kDa).

Table 2 – ERMBPs identified by LC–MS/MS from a gel area of 40–55 kDa which differed between healthy control and PV patient samples.

Protein and entry name[a]	Accession number	Mol. weight (kDa)	No. of unique peptides	Sequence coverage (%)	Mascot score
Actin, cytoplasmic 1, ACTB	P60709	41.7	11	26	229
Basigin, BASI	P35613	42.2	4	12	154
cAMP-dependent protein kinase type I-alpha regulatory subunit, KAPO	P10644	43.0	12	29	310
Dematin, DEMA	Q08495	45.5	19	49	861
Endoplasmic reticulum resident protein 44, ERP44	Q9BS26	46.9	6	13	125
Flotillin-1, FLOT1	O75955	47.4	21	51	774
Flotillin-2, FLOT2	Q14254	47.1	21	43	705
Protein disulfide-isomerase A1, PDIA1	P07237	57.1	3	9	102
Protein disulfide-isomerase A6, PDIA6	Q15084	48.1	5	14	288
Solute carrier family 2, facilitated glucose transporter member 1, GTR1	P11166	54.1	5	11	176
Thioredoxin domain-containing protein 5, TXND5	Q8NBS9	47.6	5	12	142
Tropomodulin-1, TMOD1	P28289	40.6	8	28	265
55 kDa erythrocyte membrane protein, EM55	Q00013	52.3	11	23	321
Annexin A7, ANXA7	P20073	52.7	3	5	83
26S protease regulatory subunit 8, PRS8	P62195	45.6	3	5	141
Ubiquitin-associated domain-containing protein 1, UBAC1	Q9BSL1	45.5	3	6	99

[a] 13 proteins highlighted in bold were selected for spectral counting.

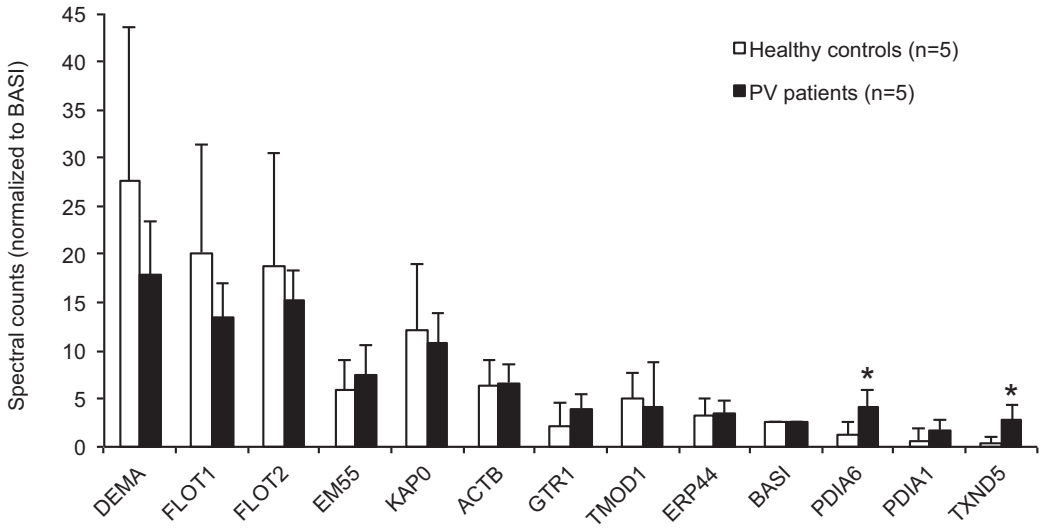

Fig. 2 – Quantification of 40–55 kDa ERMBPs by spectral counting. Basigin (BASI) was used for normalization. PDIA6 and TXND5 were up-regulated in 3.2 and 7.9 fold, respectively, in PV patients compared to healthy controls. An asterisk indicates statistical significance (Wilcoxon rank sum test, $p < 0.05$). Error bars represent SD.

counts performed on the post-refrigerated samples after 3 days revealed a reticulocytopenia.

3.2. *Identification of human ERMBPs*

ERMBPs were isolated from erythrocytes as previously described [7], and then separated by SDS-PAGE. To evaluate the performance of the isolation and identification methods, a gel lane of one of the healthy controls was cut into 14 slices. Proteins were in-gel digested and analyzed by LC–MS/MS. In total 41 proteins were identified by the Mascot database search with minimum requirements of two peptides per protein, protein and peptide confidence of 99% and 95%, respectively. The complied list of all identified proteins is shown in Supplementary Table 2. The most abundant erythrocyte associated proteins were spectrin alpha chain, spectrin beta chain, ankyrin, band 3 anion transport protein, erythrocyte membrane protein band 4.1 and erythrocyte membrane protein band 4.2. These were mostly identified in the vicinity of their known molecular weight. On the basis of their GO annotation (Scaffold), 30 of the identified proteins were membrane located/associated, i.e. assigned as ERMBPs (Supplementary Figure 1). The identification of extracellular proteins such as serum albumin, Ig gamma chain or fibrinogen gamma chain is to be expected as they may have bound to a mature erythrocyte or be present as contaminants. Even though a mature erythrocyte does not have a nucleus or other organelles, nuclear and endoplasmic reticulum associated proteins were observed in our data. In this respect many of the proteins were reported to

Table 3 – Validation of the differentially regulated ERMBPs by XIC and SRM methods.

Protein and entry name	Protein abundance ratio (PV patients/healthy controls)[a]		Wilcoxon rank sum test (p value)[b]	
	XIC data	SRM data	XIC data	SRM data
Actin, cytoplasmic 1, ACTB	0.94	1.36	0.406	0.063
Dematin, DEMA	0.57	0.96	0.031*	0.313
Endoplasmic reticulum resident protein 44, ERP44	1.64*	1.57*	0.062	0.031*
Solute carrier family 2, facilitated glucose transporter member 1, GTR1	1.41	0.95	0.906	0.500
55 kDa erythrocyte membrane protein, EM55	1.43	1.20	0.781	0.219
Protein disulfide-isomerase A1, PDIA1	1.86*	3.46*	0.094	0.031*
Protein disulfide-isomerase A6, PDIA6	2.78*	2.44*	0.031*	0.031*
cAMP-dependent protein kinase type I-alpha regulatory subunit, KAPO	1.19	1.01	0.594	0.500
Thioredoxin domain-containing protein 5, TXND5	2.79*	2.16*	0.031*	0.031*

[a] An asterisk represents ratios >1.5.
[b] An asterisk represents $p < 0.05$.

be present in multiple subcellular locations such as membrane and nucleus, membrane and endoplasmic reticulum, or membrane and cytoplasm.

3.3. *Quantification of ERMBPs by spectral counting*

In order to detect differentially regulated ERMBPs between PV patients and healthy controls, proteins from the five patients and controls were separated on gel and compared visually after silver staining. Three consecutive gels were prepared. On all the replicate gels differences in protein intensity could be detected between the PV patients and the healthy controls in the molecular mass area of 40–55 kDa (Fig. 1). Proteins from this region were cut from each sample lane, in-gel digested and analyzed by LC–MS/MS. Altogether 16 proteins were identified as membrane associated proteins in this mass range (Uniprot) with a minimum of three peptides per protein (see Table 2). Out of these ERMBPs, 13 proteins, identified in at least four of the PV patients or four of the healthy controls, were selected for quantification (see Table 2 highlighted proteins).

Peptide-spectrum matches obtained from the Mascot results were counted manually for each protein in the healthy controls and PV patients (Supplementary Tables 3 and 4). Normalization of the spectral counts was first tested with a house-keeping protein actin (Supplementary Figure 2). However, while actin was detected as an ERMBP, it is also well characterized as an abundant cytoskeletal protein. For quantifying ERMBPs, normalization with an integral membrane protein would be preferable to actin. Two of the quantified proteins are characterized as transmembrane proteins: solute carrier family 2, facilitated glucose transporter member 1 (GTR1) and basigin. GTR1 could not be used for the normalization purpose as it was not detected in two of the control samples, but basigin was observed from all the samples (Supplementary Table 4). Furthermore, when normalized with actin, the spectral counts of basigin appeared to be relatively constant between the patients and controls (Supplementary Figure 2). Basigin is present in erythrocyte membranes and a determinant for the Ok blood group system [27]. To our knowledge expression changes of basigin in the surface of mature erythrocytes has not been reported, although it has been shown to be regulated as a receptor for *Malaria falciparum* [27]. On the basis of our data and the literature, basigin was chosen as a reference protein for normalizing the quantitative data of ERMBPs in this study.

Statistical significance of protein abundance changes was obtained by applying Wilcoxon rank sum test for each protein. This is a non-parametric alternative to the two sample *t*-test, and is based solely on the order in which the observations from the two samples fall [28]. Comparisons of the healthy controls versus the PV patients revealed that protein disulfide isomerase A6 (PDIA6) and thioredoxin domain-containing protein 5 (TXND5) were significantly induced in the PV patients with ratios 3.2 and 7.9, respectively (Fig. 2). Even though the up-regulation of these proteins in the PV patient samples were detected, their spectral counts, as well as those of basigin, were relatively low compared to other common ERMBPs; dematin (DEMA), flotillin-1 (FLOT1), flotillin-2 (FLOT2), cAMP-dependent protein kinase type 1-alpha regulatory subunit (KAPO), 55 kDa erythrocyte membrane protein (EM55), actin cytoplasmic 1 (ACTB), present in Fig. 2. Therefore we chose to

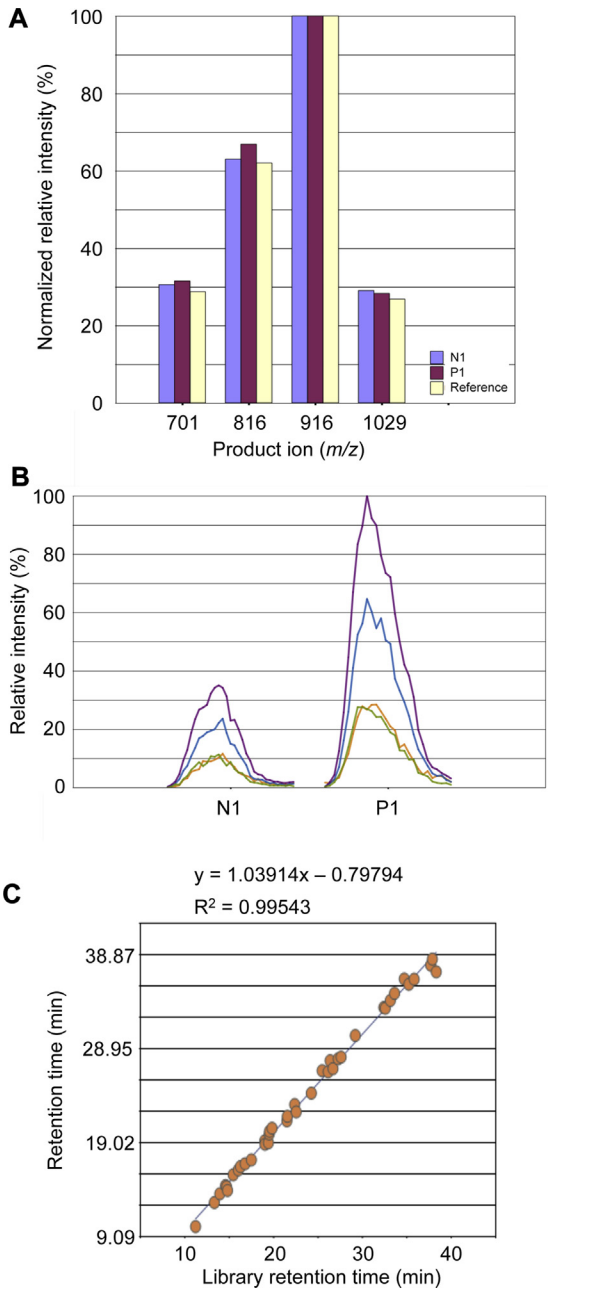

Fig. 3 – SRM assay for validating differentially regulated ERMBPs. (A) Representative data of healthy control (N1) and PV patient (P1) are shown for a peptide TGEAIVDAALSALR surrogate of the differentially regulated protein PDIA6. Four transitions belonging to the precursor ion (*m/z* 693.8829) were compared with a reference spectrum in the library generated on the Q Exactive instrument. (B) The peak profiles of the peptide were plotted against the retention time using Pinpoint software. The area under the curve was calculated and used for quantification. (C) LC retention time of the targeted peptides was plotted for comparing the library (Q Exative) and SRM (TSQ Vantage). LC conditions in both analysis using Q Exactive and TSQ included linear 40 min gradient for peptide separation. The observed linear correlation between the two LC workflows was 0.995.

Table 4 – Major functions of the ERMBPs up-regulated in PV patients.

Protein	Major functions[a]	Associated diseases/conditions	References
Protein disulfide isomerase A1	Isomerase activity Enhance protein folding Electron carrier activity Oxidoreductase activity	HIV-1 Acute myeloid leukemia Ovarian cancers	[37] [38] [39]
Protein disulfide isomerase A6	Isomerase activity Enhance protein folding Electron carrier activity Oxidoreductase activity	Chronic lymphatic leukemia Multiple myeloma Glioma Hepatocellular carcinoma Prostate cancer Cervical cancer	[40] [41] [42] [43] [44] [45]
Thioredoxin domain-containing protein 5	Isomerase activity Enhance protein folding Electron carrier activity Oxidoreductase activity Anti apoptotic activity Cellular membrane organization Belongs to the pathway of membrane trafficking	Gastric cancer Lung cancer Hepatocellular carcinoma Androgen independent prostate cancer Colorectal adenoma and cancer	[46] [47] [48] [49] [50]
Endoplasmic reticulum resident protein 44	Isomerase activity Enhance protein folding Electron carrier activity Oxidoreductase activity Participate in the glycoprotein metabolism	Lipid homeostasis in cardiovascular disease and type 2 diabetes	[29]

[a] UniprotKB/Swiss-Prot database (http://www.uniprot.org/uniprot).

verify the quantification results using precursor ion intensities.

3.4. *Quantification of differentially regulated ERMBPs based on precursor ion intensities*

Based on the spectral counting results we selected eight proteins, DEMA, EM55, KAPO, GTR1, ERP44, PDIA6, PDIA1 and TXND5 for further quantification. Actin, as well as basigin, was included as house-keeping proteins. Precursor ion intensities of all the identified unique peptides were measured on XICs, which were summed up for each protein and normalized to basigin. The protein abundance changes and statistical significance (Wilcoxon rank sum test) between PV patients and healthy controls are shown in Table 3. The XIC intensity based quantification results were in keeping with the spectral counting results (refer to Fig. 2). PDIA6 and TXND5 showed significantly increased levels (approximately 2.8 fold) in the PV patients. Even though the changes in PDIA1 and ERP44 were not statistically significant ($p > 0.05$), increases of 1.9 and 1.6 fold, respectively, were indicated in the patients. Although DEMA was observed at a significantly higher level in the healthy controls than in the PV patients, it is noted that it is highly abundant in the erythrocyte cytoskeleton and this finding could be compromised.

3.5. *Validation of the differentially regulated ERMBPs using SRM*

To validate the precursor intensity based quantification results, the same erythrocyte membrane samples were in-solution digested and measured by SRM analysis. The intensity ratios of all primary and secondary transitions in the iSRM measurements were compared with the spectral library to assure that the major fragment ions were matched. Peptides with Dot Product correlation coefficients (in Pinpoint) above 0.85 were accepted. For quantification four transitions per peptide and three unique peptides per protein were monitored without using the iSRM functionally. Finally 113 transitions for 36 precursor ions were used in quantification (see Supplementary Table 1). The in-solution digests of the PV patient and healthy control samples were randomized and analyzed in duplicate. No isotope labeled standard was used, but only semi-quantitative relative quantitation was performed.

Fig. 3 illustrates data registered in an SRM experiment with an example peptide "TGEAIVDAALSALR" (m/z 693.8829, 2+), which is a surrogate of the differentially regulated protein PDIA6. The peptide was eluted from the column at 38.47 and at 38.19 min in healthy control (N1) and PV patient (P1), respectively. The relative intensity of the four transitions, m/z 701.43, 816.46, 915.53, and 1028.61, measured for the precursor ion in the healthy control and PV patient samples were in agreement with a reference spectrum in the library (the Dot Product correlation coefficient was 0.99) (Fig. 3A). Absolute intensities were compared, all the transitions were higher in PV patient (P1) than healthy control (N1) (Fig. 3B). The additional verification for the peptides identity was a good correlation of their retention times with the spectral library (see Fig. 3C).

The results from the SRM experiment (in-solution digests) and from the data dependent runs (in-gel digests) are summarized in Table 3. Most of the SRM data (ratio of PV patient to

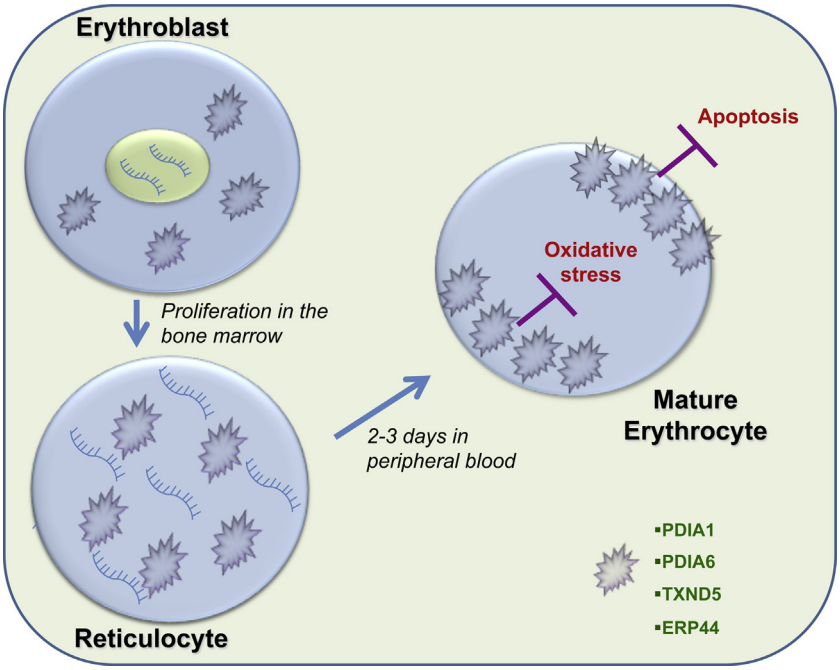

Fig. 4 – Up-regulation of various PDI family proteins could be involved in the prolonged survival of PV erythrocytes. PDIA1, PDIA6, ERP 44 and TXND5 reside in ER of immature erythroblasts and reticulocytes, and may be incorporated in mature erythrocytes. Erythrocytes are exposed to one of the highest levels of oxidative-stress conditions in the body and thioredoxin domains characteristically tolerate oxidative stress. Since all of those PDI family proteins have 2–3 thioredoxin domains they can maintain oxidative stress response functions vital for erythrocytes survival. In addition TXND5 has an anti-apoptotic function and up-regulation of TXND5 in PV patients may enhance in the prolonged survival of erythrocytes.

healthy control) correlate with the XIC data. The abundance of two proteins, PDIA6 and TXND5, increased in the patient samples in both the experiments. These proteins represented more than 2 fold increased values while ERP44 had about 1.6 fold increase, which were similar to the XIC data. PDIA1 showed about 3.5 fold increase in the PV patients. Wilcoxon rank-sum test confirmed all the above results ($p < 0.05$), i.e. the increase of PDIA6, TXND5, ERP44 and PDIA1 were statistically significant (Table 3). None of other proteins measured showed significant difference.

The four proteins up-regulated in the PV patients belong to the PDI protein family. The major functions and associated diseases of the four PDIs are summarized in Table 4. PDIs usually reside in the endoplasmic reticulum (ER), although they can be released to function at the cell surface or extracellular matrix [29]. PDIs function as molecular chaperones and as disulphide oxidoreductase/isomerases during protein folding. The PDI family features two conserved thioredoxin domains, including an active motif (CGHC) of the disulfide redox response [30,31], and is involved in oxidative stress response [23]. In 2010, Vener et al. found increased oxidative stress in primary and post PV myelofibrosis [32]. Oxidative stress is a known up-regulator of thioredoxin expression [31] that provides protection against the stress and has anti-apoptotic effects [33]. Although non-nucleated mature erythrocytes were considered to be eliminated by mechanisms other than apoptosis, erythrocyte apoptosis has currently been well acknowledged in the field [34–36]. In that context, increased abundance of the 4 PDI proteins having thioredoxin domains may prevent oxidative stress and apoptosis in erythrocytes of PV patients (Fig. 4).

4. Conclusion

Quantitative proteomics revealed that ERMBPs, PDIA6, TXND5, ERP44 and PDIA1, were up regulated in the PV patients with the JAK2 V617F mutation when compared to the healthy controls. Since these four PDI proteins could be considered as an inhibiting factor of oxidative stress and apoptosis, they may be associated with the increased red cell mass, which is a cardinal feature in PV (Fig. 4). To our knowledge this is the first time to shed light on the membranes in PV patients. These initial findings appear interesting enough to warrant a larger study of erythrocyte membrane proteins in PV patients. It will be prudent to carry out this work in patients both treated and not treated with hydroxycarbamide, and/or in vitro experiments with and without hydroxycarbamide treatment, to negate the effects of the drug on these findings. If it is confirmed that the PDI proteins are in fact differentially regulated in PV patients, there may be a potential for targeted therapy. On the other hand, we expect that, as the JAK2 mutation in PV patients activates the kinase activity, phosphoproteomics approach will also provide new insights into the mechanism of the disease.

Conflict of interest

The authors declare no competing financial interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2015.04.001.

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