รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษากลไกของ Unfolded protein response ในผู้ป่วยโรคธาลัสซีเมียชนิดฮีโมโกลบิน เอชคอนสแตนท์สปริง

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เมษายน 2562

สัญญาเลขที่…MRG5980160…

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

เอกสารแนบหมายเลข 2/1

บทคัดย่อ

รหัสโครงการ: MRG5980160

ชื่อโครงการ: การศึกษากลไกของ Unfolded protein response ในผู้ป่วยโรคธาลัสซีเมียชนิดฮีโมโกลบินเอชคอนสแตนท์สปริง

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ระยะเวลาโครงการ: 2 พ.ค. 2559 ถึง 1 พ.ค. 2561

บทคัดย่อ:

โรคธาลัสซีเมียเป็นโลหิตจางถ่ายทอดทางพันธุกรรมที่พบบ่อยในประเทศไทย โรคนี้มีสามเหตุจากความบกพร่องในการสังเคราะห์สายกลบินส่งผลทำให้มีการสะสมสายโกลบินส่วนเกินในเซลล์เม็ดเลือดแดงมากผิดปกติ การศึกษานี้ได้นำเทคนิคโปรติโอมิกส์มาใช้ในการตรวจหาการเปลี่ยนแปลงระดับการแสดงออกของโปรตีนที่อยู่ในเซลล์เม็ดเลือดผู้ป่วยธาลัสซีเมีย จากการเปรียบเทียบระดับโปรตีนในเม็ดเลือดแดงผู้ป่วยโรคธาลัสซีเมียชนิดฮีโมโกลบินเอชคอนสแตนท์สปริงกับคนปกติพบการเพิ่มขึ้นของโปรตีนที่เกี่ยวข้องกับกลไล Unfolded protein response เช่น โปรตีน HSP70, GRP78 and Calreticulin

นอกจากนี้การศึกษานี้ยังได้นำเทคนิคโปรติโอมิกส์มาตรวจหาระดับโปรตีนในเกล็ดเลือดที่ส่งผลต่อการกระตุ้นเกล็ดเลือดและปัจจัยการแข็งตัวของเลือดในกลุ่มผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี ผลการทดลองพบว่าระดับการกระตุ้นของเกล็ดเลือดและความเข้มข้นของ prothrombin fragment 1+2 ในผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอีเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับคนปกติ ผลการวิเคราะห์โปรตีนรวมพบโปรตีนแสดงออกแตกต่างกันจำนวน 23 ชนิด ประกอบด้วยโปรตีนโครงสร้างในเกล็ดเลือด 13 ชนิด เช่น F-actin -capping protein subunit beta, Actin -related protein 2/3 complex sub unit 5, PDZ and LIM domain protein 1, Trangelin2, Tropomyosin และ Myosin นอกจากนี้ยังพบการแสดงออกของโปรตีนที่เกี่ยวข้องกับการสร้างพลังงานภายในเซลล์ 1 ชนิด (Pyruvate kinase PKM 2) โปรตีนที่เกี่ยวข้องกับการกระตุ้นระบบภูมิคุ้มกันในเซลล์ 1 ชนิด (Beta-2-Microglobulin) โปรตีนที่เกี่ยวข้องกับการสังเคราะห์ฮีม (Biliverdin reductase A และ Hemoglobin beta subunit) และเอนไซม์ต้านอนุมูลอิสระ 2 ชนิด (Peroxiredoxin 6 และ Glutathione S transferase P) ระดับของโปรตีน chaperone (Hsp70), fibrinogen receptor (integrin **α**IIb) และ chemokine (PF4)

การศึกษากลไล Unfolded protein response ในผู้ป่วยฮีโมโกลบินเอชคอนสแตนท์สปริง และการค้นพบโปรตีนที่เกี่ยวข้องการกระตุ้นเกล็ดเลือดและสภาวะการแข็งตัวของเลือดสูงเกินปกติในผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี ทำให้เข้าใจกลไกการเกิดพยาธิสภาพในเซลล์เม็ดเลือดผู้ป่วยโรคธาลัสซีเมียเพิ่มขึ้น

คำหลัก : ธาลัสซีเมีย, ฮีโมโกลบินเอช, เบต้า-ธาลัสซีเมีย, เทคนิคโปรติโอมิกส์

เอกสารแนบหมายเลข 2/2

Abstract

Project Code : MRG5980160
Project Title : Investigation into the unfolded protein response pathway in HbH-CS erythroblast
Investigator : Dr.Kamonlak Leecharoenkiat
E-mail Address : rbc_2524@hotmail.com
Project Period : 2 May 2559- 1 May 2561

Abstract:

Thalassemia is the most common inherited blood disease detected in Thai population. Increasing accumulation of excess globin chain inside thalassemic red blood cells resulted in many abnormal pathways. In this study, the proteomic analysis was used as a tool used to identify the changed in cellular proteins in thalassemia diseases. Comparing RBC proteins between normal and HbH-CS patients revealed the up-regulation of protein related to the UPR pathway (HSP70, GRP78 and Calreticulin).

Moreover, we also performed the proteomic analysis to identify the platelet proteome related to platelet activation and hypercogulable state in the β-thalassemia/HbE. We found that the levels of platelet activation and prothrombin fragment 1+2 in β- thalassemia/HbE patients were significantly increased as compare to normal controls ($p<0.05$). Proteomic analysis revealed a total of 23 differentially expressed proteins, including 13 platelets cytoskeleton proteins such as F-actin -capping protein subunit beta, Actin -related protein 2/3 complex sub unit 5, PDZ and LIM domain protein 1, Trangelin2 and subunits of Tropomyosin and Myosin, A protein is directly involved in production of intracellular energy (Pyruvate kinase PKM), A protein were immune activation (Beta-2-Microglobulin), 2 proteins are involved heme synthesis (Biliverdin reductase A and Hemoglobin beta subunit) and 2 proteins are antioxidant enzyme (Peroxiredoxin 6 and Glutathione S transferase P). A protease inhibitor (Leukocyte elastase inhibitor) had a significantly decreased expression in β-thalassemia/HbE.

In conclusion, the study of UPR pathway in HbH-CS may lead to a better understanding the biological pathogenesis underlying clinical presentation of the HbH-CS patients. The platelet proteins related to platelet activation and the hypercoagulable state of β-thalassemia/HbE have now described. The data identified from this study will lead some novel insight to understand the pathophysiological conditions in the thalassemia patients.

Keywords : Thalassemia, HbH disease, beta thalassemia, UPR response pathway, Proteomic analysis

Executive summary

Thalassemia is the most prevalent and widely distributed inherited blood disordered detected in Thailand. The HbH results from three α globin genes deletion (--/-α) which leave only one functional α-globin gene. Approximately 20% of patients with HbH disease are caused by compound heterozygous α-thalassemia 1 and the variants α-globin gene, such as Hb Constant Spring (HbH-CS), Pakse or Quong Sze mutations. HbH-CS is the most severe form of α-thalassemia syndrome in Thai population. The HbH-CS results from the co-inheritance of α^0-thalassemia together with a common non-deletional hemoglobin Constant Spring (HbCS). The HbCS is caused by a mutation at the termination condon of α-globin gene leading to 31 additional amino acid residues attached to the C terminal end of the α -globin. Patients who carry HbH-CS (--/$\alpha^{cs}\alpha$) normally have clinically more severe than patients with a common HbH disease (--/-α). The pathogenesis of HbH is hemolysis of erythrocyte resulting from the effect of excess γ and β globin, however, the precise mediator or mechanism by which this occurs has yet to be characterized. This study sought to determine whether there were alterations in the RBC pathways of HbH-CS other than these abnormalities. Proteomic analysis of RBC proteins comparing between normal and HbH-CS patients was analyzed. The analysis of UPR pathway was conducted on erythroblasts cultured from HbH-CS patients compared to erythroblasts cultured from normal subjects. The analysis undertaken included determination of cellular proliferation, cell apoptosis by flow cytometry and expression of UPR related genes (XBP1, CHOP, ERDJ4, EDEM) observed by RT-PCR.

β-thalassemia/HbE is an inherited hemolytic anemia caused by defect in β-globin synthesis resulting in accumulation of excess α-globin chains in red blood cells. A hypercogulable state leading to high risk of thromboembolic event, is one of the most common complications observed in this disease, particularly in patients with splenectomy. Previous studies suggested that increased platelet activation and coagulation factors in β-thalassemia/HbE intermediate patients promote the hypercoagulable state. However, the hypercoagulable state as well as the molecular mechanism regarding this pathogenesis in β-thalassemia/HbE is not yet well understood. Thus, this study aimed to identify proteins related to platelet activation

and to hypercoagulable state in the β-thalassemia/HbE patients. These results will improve our understanding of the biological pathogenesis of those two diseases.

Objectives

1. To analyse RBC proteome and the UPR pathway in HbH-CS compared to normal controls.
2. To investigate platelet proteins related to platelet activation and hypercoagulable state in the β-thalassemia/HbE patients.

Method

HbH-CS patients and normal subjects

All HbH-CS samples were obtained from Nakhonpathom hospital, Thailand. The study was approved by the Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University. Complete blood counts and RBC indices of both normal controls and β°-thalassemia/HbE patients were determined using an automated cell counter (ADVIA210, Bayer, Tarrytown, NY, USA). Hemoglobin typing was analyzed by an automatic HPLC system (VARIANT™, BioRad, Hercules, CA, USA). All normal controls were screened to be normal for red blood indices and hemoglobin by CBC and hemoglobin typing analysis.

Red blood cell proteome analysis

Packed red cells proteins were separated from 8 HbH-CS and 8 normal subjects. The protein profiling was analyzed using two-dimensional gel electrophoresis. The differential protein spots were identified using LC/MS/MS analysis and validated by western blot analysis.

$CD34^+$ hematopoietic stem cell isolation and Erythroblast culture

CD34+ hematopoietic progenitor cells was isolated from 30 mL peripheral blood by using positive immunomagnetic selection method according to the manufacturer's protocol. Briefly, The whole blood was centrifuged at 600 x g for 8 min to separate the plasma. After discarding the plasma, packed cells were diluted with two volumes of 1xPBS buffer and carefully overlaid on Lymphoprep^{TM} (1.077 density) with a ratio 2:1 in total volume of 30 mL. The suspension was centrifuged at

800 x g for 20 minutes at 22 ºC in a swinging-bucket rotor centrifuge. The mononuclear cell layer was then collected in a new tube and washed with 1xPBS buffer and selected by magnetic column. The selected CD34+ cells was cultured under appropriate cell culture conditions for driving erythroid proliferation and differentiation following method explained in our previous study. The CD34+ cells was grown for 14 days in Iscove's modified Dulbecco medium supplemented with 15% heat-inactivated fetal calf serum, 15% human AB serum, and three cytokines including 2 U/mL rhEPO, 20 ng/mL rhSCF and 10 ng rhIL-3 at 37 oC in a high-humidity, 5% CO2.

Apoptosis detection by flow cytometry

2 x 10^5 of cultured erythroblasts were washed with 1 ml of 1x PBS. After centrifugation at 5,000 rpm for 5 min, the supernatant was discarded. The cell pellet was resuspended with 400 ul of 1x PBS and incubated with 1 µL of FITC-conjugated Annexin V and 5 µL propidium iodide (PI) for 15 min. Fluorescence signals were detected using a FACSCalibur flow cytometer.

RNA extraction

2x10^6 erythroblasts were homogenized with 750 µL of Isogen-LS reagent by vortexing and pipetting. 200 µL of chloroform was added and the suspension was shacken vigorously. The aqueous phase was achieved after centrifuged at 14,000 rpm for 15 min at 4 °C. 450 µL of the aqueous phase was transferred to a new tube and 400 µL of Isopropanol was added into the solution before storing for 10 min at room temperature. The solution was then centrifuged at 14,000 rpm for 10 min and supernatant was removed. The RNA pellet was washed with 1 mL of 70% ethanol and centrifuged at 14,000 rpm for 5 min. The supernatant was completely removed and the RNA pellet was dried in vacuum drier for 7 min. The RNA pellet was re-solubilized with 15 µL Rnase water and stored at -20 °C until used.

Reverse Transcription PCR to detect expression of XBP1 and genes related to UPR pathway

The genes related to UPR pathway of cultured erythroblasts were observed. 1 µg RNA was amplified by one step RT-PCR with specific primer for XBP1, CHOP**,**

EDEM, ERdj4 and actin according to the manufacturer's instructions (OneStep Ahead RT-PCR Kit, Qiagen).

Detection of platelet activation by flow cytometry

The platelet activation was measured in both whole blood and isolated platelet fractions. The whole blood or isolated platelet fractions were inhibited by 2µM of Prostaglandin E1 (PGE1, Clayman Chemical, Michigan, USA). 1 µL of anti-human CD41-FITC, 1 µL anti-human CD62P-PE and 1 µL anti-human GPA-APC was added to the inhibited samples and analyzed by FACs Calibur (BD Bioscience, USA). The platelet population was determined as CD41-positive and activated platelets were identified as CD41-positive/CD62P-positive. Contaminated RBC in the platelet fraction was determined as GPA-positive.

Platelet proteome analyzed by Two dimensional gel electrophoresis (2-DE)

The platelet proteins were lysed with 150 µL of protein lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, and 1% human protease inhibitor cocktail. The platelet protein was solubilized at 4 °C for 24 hrs. The proteins were rehydrated for 14 hours in reservoir slots of a re-swelling tray and isoelectric focusing was examined under horizontal apparatus, Ettan IPGphore3 (GE Healthcare). After the equilibration step was completed, the proteins were electrophoresed in the second-dimension using 12.5 % vertical SDS-PAGE in a MiniVE vertical electrophoresis system (GE Healthcare). Gels were stained with 0.1% Coomassie Brilliant Blue G250 in 40% methanol for 24 hours and destained with MilliQ water for 6 hours to visualize the protein spots. The stained 2D gels were scanned under visible light at 600 µm/pixel resolution. Image data were analyzed using Image Master V.7 software (GE Healthcare).

Western blot analysis

Thirty milligrams of RBC or platelet protein samples were separated on polyacrylamide gel. After separation, the platelet proteins were transferred and blotted onto 0.2 mM pore nitrocellulose membranes (GE Healthcare) using Semi-Dry Blotter (10 x 10 cm) for Mini System (Cleaver Scientific, United Kingdom) and subsequently blocked with 5 % non-fat milk in TBS/0.05 % Tween (TBST) for an hour at room temperature, then washed with TBST for 10 min and repeated three times. The

membranes were probed with an appropriate dilution of primary and secondary antibodies which were diluted with 5 % BSA. After incubation, the signal was developed by adding Chemiluminescense ECL (Pierce) substrate and exposed with X-ray film.

Results

Clinical data of subjects

4 HbH-CS and 4 normal subjects was included in this study. Approximately 30 mL of peripheral blood of HbH-CS patients and normal subjects was collected after Ethical approval and individual informed consent. Diagnosis of HbH-CS was performed based on Hb typing and PCR-sequencing analysis. The HbH-CS patients showed a significantly lower Hb, Hct, MCV, MCH and MCHC levels while the reticulocyte count of HbH-CS was significantly higher than normal controls ($p<0.05$) (Table 1).

Table 1. Clinical parameters of normal subjects and HbH-CS patients

Clinical parameter	Normal subjects (n=4)	HbH-CS (n=4)
Gender (Male/ Female)	2/2	1/3
RBC count (109 cells/ul)	4.45+0.55	4.57+0.75
Hb (g/dL) *	13.32±1.65	8.21±0.78
HCt. (%) *	39.00±4.15	32.78±3.50
MCV (fl) *	85.88±4.80	72.22±6.44
MCH (pg) *	29.26±1.51	19.01±2.03
MCHC (g/dL) *	34.08±0.71	25.31±2.45
RDW (%) *	12.75±0.78	20.32±3.11
Reticulocyte (%) *	1.13±0.22	5.37±1.92

*Statisticaly significant ($p<0.05$)

Comparative red blood cell proteome between HbH-CS and normal subjects

A total of 20 differentially expressed spots were detected of which 10 spots were up regulated and 10 were down regulated (Table 2). Down regulation of 3 RBC membrane proteins (Ankyrin, Topomyosin and Integrin) and up regulation 3 of chaperone or unfolded protein response proteins (HSP70, GRP78 and Calreticulin) in HbH-CS was detected.

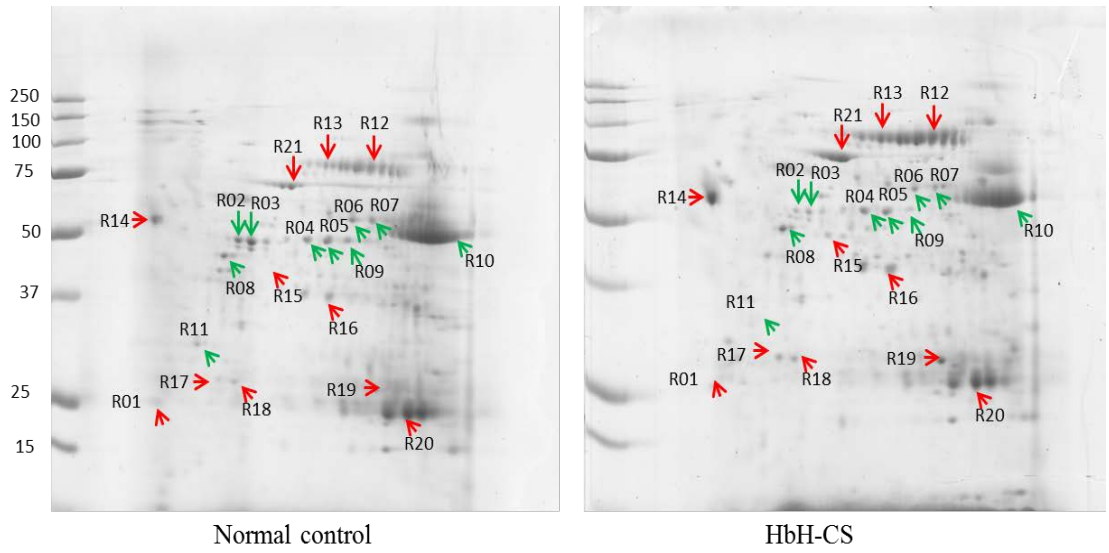

Figure 1. Representative 2DE of RBC proteins. Differential expression of RBC proteins comparing between normals and HbH-CS patients. A total of 20 significantly different protein spots with 10 up- regulated proteins (red arrow) and 10 down regulated proteins (green arrow) were detected.

Table 2. List of 20 differential spots with 19 differential RBC proteins compared between normals and HbH-CS patients

No	Name of protein	Score	% coverage	Normal/ HbH	Cellular localization
R01	Immunoglobulin J chain	99	3	Up regulation	Blood microparticle
R02	Fibrinogen gamma chain	376	19	Down regulation	Cell surface
R03	Fibrinogen gamma chain	438	16	Down regulation	Cell surface
R04	Integrin alpha-E	553	37	Down regulation	Cell membrane
R05	Heme oxygenase 2	58	16	Down regulation	Cytoplasm
R06, 07	Beta-2-glycoprotein	42	16	Down regulation	Cell surface
R08	78 kDa glucose-regulated protein	696	73	Up regulation	Endoplasmic reticulum membrane
R09	Fibrinogen beta chain	172	13	Down regulation	Cell surface
R10	Ankyrin-1	172	13	Down regulation	Cytoplasm, cytoskeleton
R11	Tropomyosin alpha-4 chain	102	13	Down regulation	Cytoplasm, cytoskeleton
R12	Ig mu chain C region	325	34	Up regulation	Blood microparticle
R13	C4b-binding protein alpha chain	46	10	Up regulation	Plasma membrane
R14	Calreticulin	141	29	Up regulation	cell membrane
R15	Proliferation-associated protein	115	24	Up regulation	Cytoplasm
R16	Heat shock 70 kDa protein	363	20	Up regulation	Cytoplasm
R17	Ig kappa chain V-IV region	110	3	Up regulation	
R18	Hemoglobin subunit beta	81	4	Up regulation	Cytoplasm
H19	Carbonic anhydrase 1	122	6	Up regulation	Cytoplasm
R20	LIM domain and actin-binding protein	40	1	Up regulation	cytoskeleton

Erythroid cells expansion capacity

The number of proliferative cultured erythroblasts after cultured for 14 days was investigated by direct cell counting. The ratio of expanded erythroid cells on days 3 to day10 of 4 normal subjects and 4 HbH-CS patients was calculated. The expansion rate of HbH-CS erythroblasts was significantly decreased when compared to normal erythroblasts ($p < 0.0469$) (Figure 3).

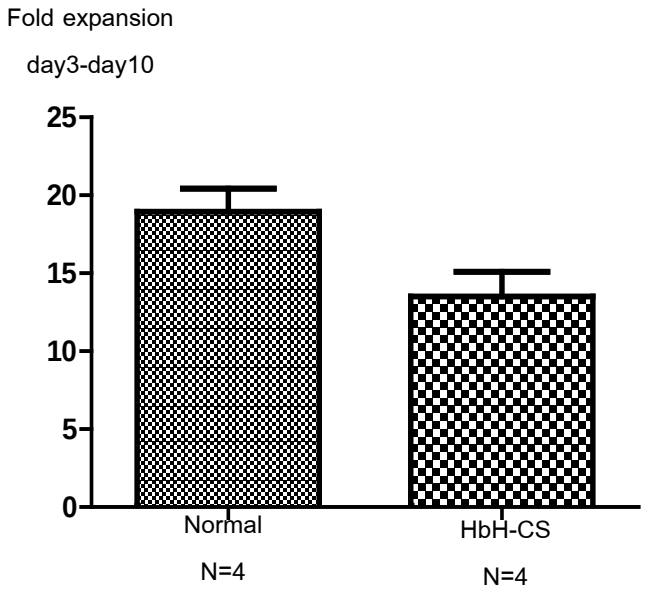

Figure 2. Fold expansion of normal subjects and HbH-CS erythroblasts during culture period. The expansion of erythroblasts calculated from day 3 to day 10 of 4 normal controls and 4 HbH-CS patients established by direct cell counting.

Determination of cell viability and apoptosis of cultured erythroblast

Erythroblasts at day 10 of culture from 4 normal controls and 4 HbH-CS patients were analyzed for apoptosis by flow cytometry using annexin V(AnnV) and propidium iodide (PI) staining. Apoptotic cells were calculated by combining early stage apoptotic cells (AnnV+/PI-; LR) and late stage apoptotic cells (AnnV+/PI+; UR) as shown in Figure 4. H-CS erythroblasts showed higher level of apoptosis than

normal control erythroblasts. About 2 folds of apoptotic cells were formed on day 10 (10.87 ± 0.53% in Hb H-CS erythroblasts and 5.33 ± 0.98 % in normal controls)

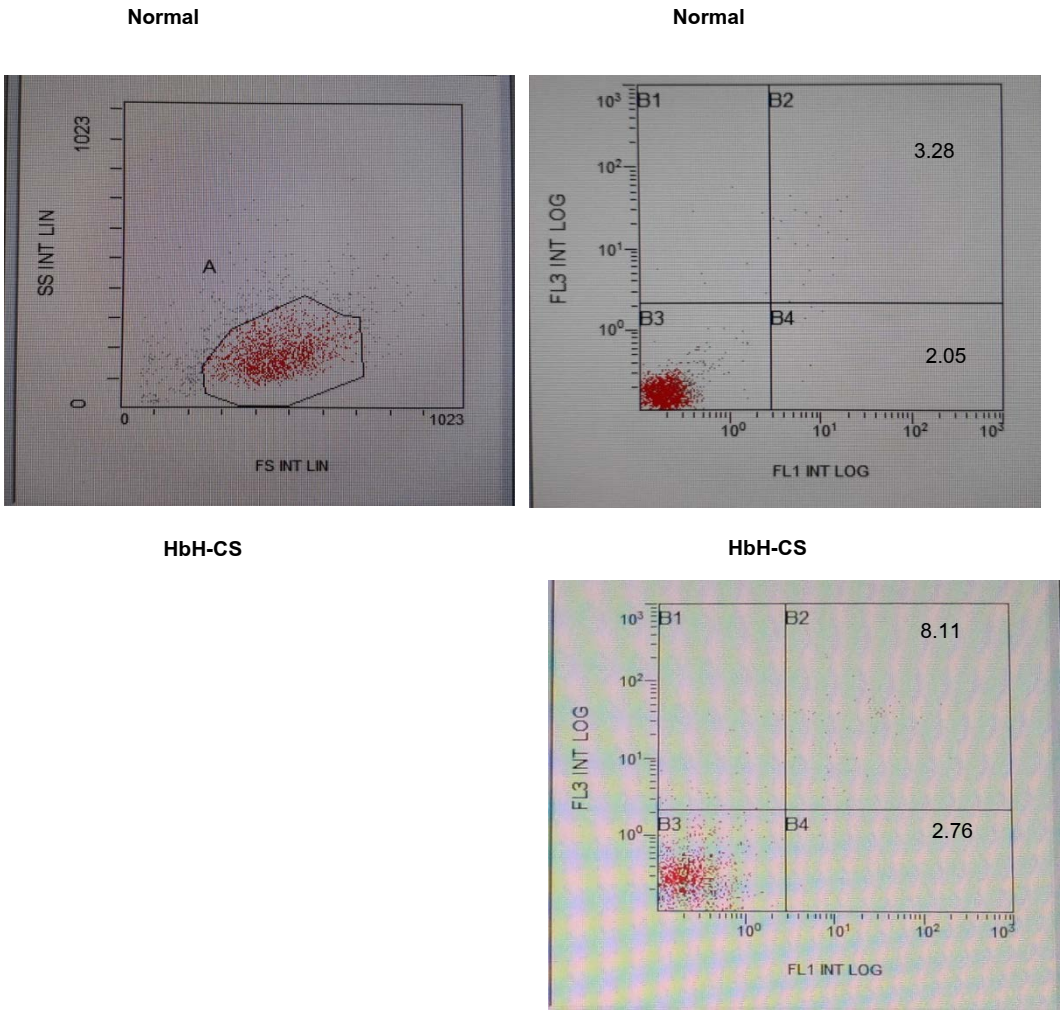

Figure 3. Dot plot of cultured erythroblasts analysed by Flow cytometry using Annexin V and PI staining. Viable cells (AnnexinV-/PI-) were detected in lower left region (LL), early apoptotic cell (AnnexinV+/PI-) in lower right region (LR) and late apoptotic cells (AnnexinV+/PI+) in upper right region (UR).

Detection of UPR response genes

Activation of the UPR was conducted by detecting the stress induced alternative splicing of the XBP-1 transcript by IRE-1 using primers able to detect simultaneously the presence of both spliced (stress induced; 416bp PCR product) and

un-spliced (unstressed; 442 bp PCR product) forms of the XBP-1 transcript Figure 5. . The obtained cDNA was amplified by specific primer of XBP-1 gene. The PCR products were visualized by 3 % agarose gel electrophoresis. The expression of three XBP-1-dependent UPR target genes including ERdj4, EDEM and CHOP was detected by RT-PCR analysis by using specific primers and the result is shown in Figure 6.

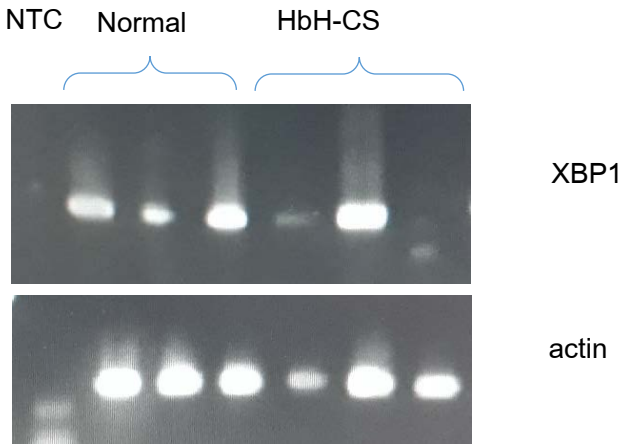

Figure 4. RT-PCR analysis of induction of XBP-1 splicing in day 10 erythroblasts from 3 normal control and 4HbH-CS.

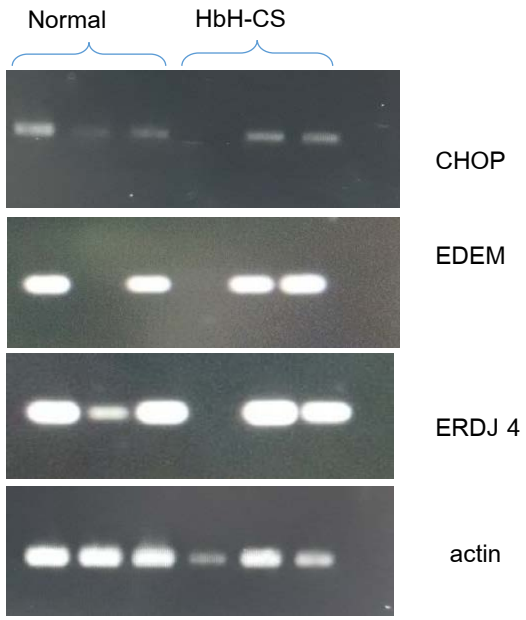

Figure 5. RT-PCR analysis of UPR related genes in day 10 erythroblasts from 3 normal control and 4HbH-CS.

Levels of platelet activation analyzed by flow cytometry

Platelet activation levels in β-thalassemia/HbE patients were determined by analyzing level of GP IIb/IIIa (CD41) and P-selectin (CD62P) positive cells. The FSC-H and SSC-H scatter gram showed populations of RBCs, platelets, and MPs or debris fractions. The percentage of platelet population (CD41-positive) in splenectomized β- thalassemia/HbE patients (86.9±4.9) was significantly higher than those of non-splenectomized β-thalassemia/HbE patients (77.1±5.6%) and normal controls (74.0±7.2%). Moreover, the splenectomized β-thalassemia/HbE patients showed a significantly higher percentage of platelet activation (CD62P-positive) (17.7±4.2 %) when compared to non-splenectomized β-thalassemia/HbE patients (6.4±1.6%) and normal controls (3.1±0.7%).

Platelet proteome of normal and β-thalassemia/HbE patients

Platelet protein expression of 4 non-splenectomized β-thalassemia/ HbE patients and 4 normal controls were analyzed by 2D gel electrophoresis. The results showed a total of 20 differentially regulated protein spots of which 19 were up-regulated and 1 protein was down-regulated in non-splenectomized β-thalassemia/ HbE patients as compared to normal controls (Figure 6) . The proteins were identified by searching the generated spectra against the database and showed expression of 22 platelet proteins from 20 protein spots as indicated in Table 3. The down-regulated proteins were defined as Leukocyte elastase inhibitors. The largest up-regulated groups were cytoskeleton membrane proteins (F-actin -capping protein subunit beta, Myosin light polypeptide 6, Myosin regulatory light chain 12A, Myosin-9, Rho GDP-dissociation inhibitor 2, Transgelin- 2, PDZ and LIM domain protein 1 and Tropomyosin alpha- 4 chain Actin -related protein 2/ 3 complex subunit 5 and Tropomyosin alpha- 1 chain) . Moreover, other proteins were a chaperone protein (Heat shock protein 70 kDa protein), a fibrinogen receptor (IntegrinαIIb), an immune cell activation (Beta-2-Microglobulin) , globin protein (Hemoglobin subunit beta) , heme synthesis protein (Biliverdin reductase), Glycolysis (Pyruvate kinase PKM) and antioxidant protein (Peroxiredoxin 6, Glutathione S-transferase P) , while platelet activation factor (platelet factor 4) was the highest expression as 5 time in β-thalassemia/HbE patients when compared to normal controls.

Table 3. Differential proteins cpmpared between non-splenectomized β-thalassemia/HbE patients and normal controls

No	Protein name	MW(Da)	PI	Score	%Coverage	β/HbE Vs normal control Fold change	Function
1	Heat Shock 70kDa protein	70294	5.48	1033	25	1.41	- Chaperone protein
2	Integrin Alpha-IIb	114446	5.21	853	11	1.36	- Fibrinogen Receptor - Platelet activation and aggregation
3	Leukocyte Elastase Inhibitor	42829	5.9	2557	44	-1.11	- Serine protease inhibitor
4	F-actin -capping protein subunit beta	31616	5.36	2034	39	1.16	- Cytoskeleton
5	Beta-2-Microglobulin	13820	6.06	42	16	1.12	- Immune activation
6	Myosin light polypeptide 6	17090	4.56	397	21	1.45	- Cytoskeleton
7	Peroxiredoxin 6	25133	6	2187	76	1.05	- Antioxidant enzyme
8	Myosin-9	227646	5.5	675	7	1.09	- -Cytoskeleton
9	Myosin regulatory light chain 12A	19839	4.67	527	41	1.06	- Cytoskeleton
	Myosin regulatory light polypeptide 9	19871	4.8	503	23	1.06	- Cytoskeleton
10	Biliverdin reductase A	33692	6.06	203	26	1.09	- Heme synthesis
	Pyruvate kinase PKM	58470	7.96	362	12	1.09	- Glycolytic enzyme
11	Actin -related protein 2/3 complex sub unit 5	16367	5.47	1147	47	1.32	- Actin polymerization
12	Glutathione S-transferase P	23569	5.43	732	18	1.18	- Antioxidant enzyme
13	Rho GDP-dissociation inhibitor 2	23031	5.1	1229	30	1.22	- Actin reorganization
14	Tropomyosin alpha- 1 chain	32746	4.69	134	13	1.04	- Cytoskeleton
15	Transgelin- 2	22548	8.41	3391	64	1.40	- Cytoskeleton
16	Hemoglobin subunit beta or Hemoglobin E	16102	6.75	985	58	3.90	- Globin
17	Platelet factor 4 (CXCL4 or PF4)	14171	9.04	792	45	5.42	- Chemokine - Platelet activation - heparin binding protein
18	PDZ and LIM domain protein 1	36505	6.56	2118	48	1.02	- Cytoskeleton
19	PDZ and LIM domain protein 1	36505	6.56	2590	44	1.05	- Cytoskeleton
20	Tropomyosin alpha- 4 chain	28619	4.67	889	57	1.09	- Cytoskeleton

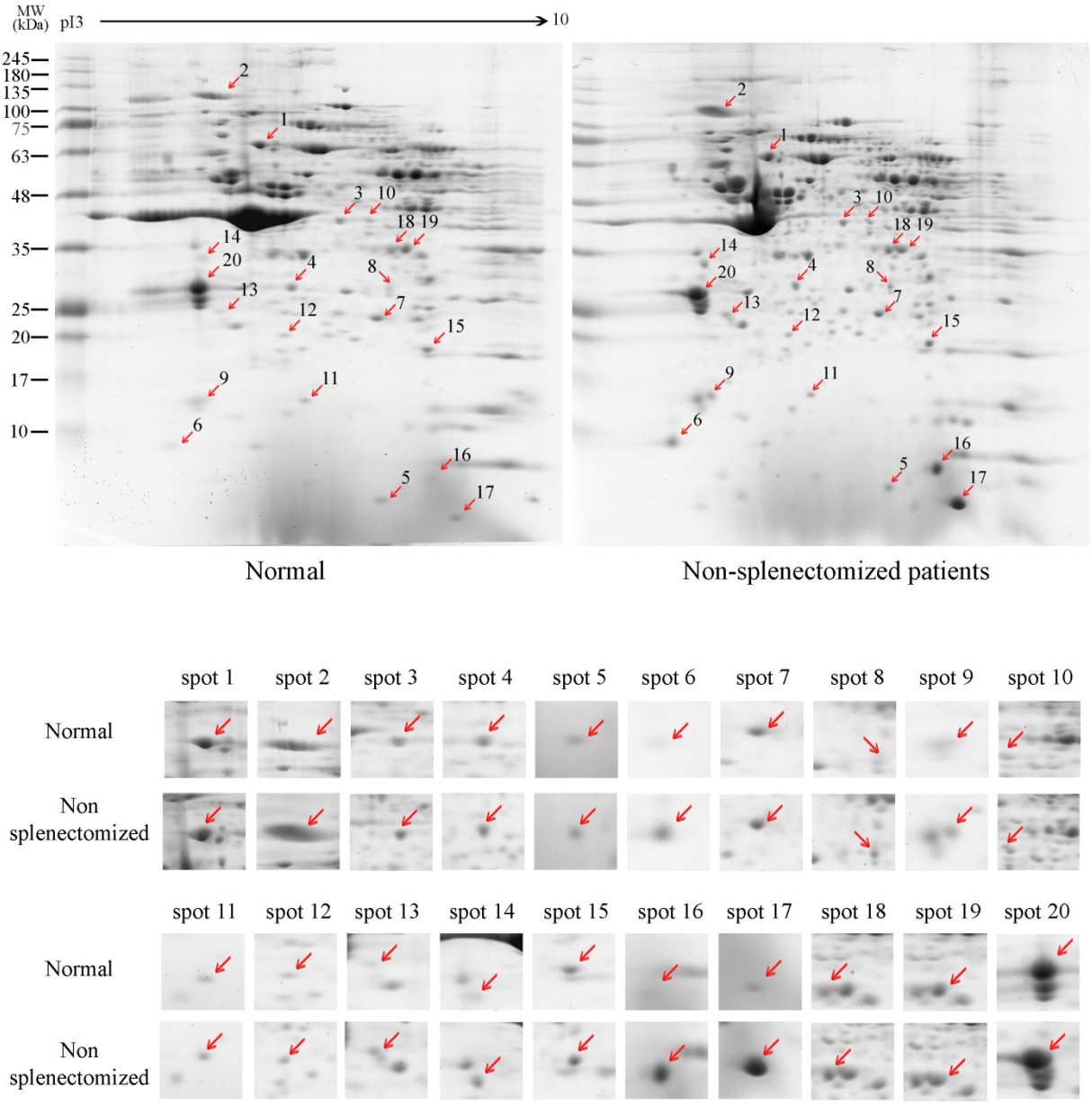

Figure 6. 2DE spots compared between normal controls and non-splenectomized β-thalassemia/HbE patients.

Representative 2D PAGE gel of platelet proteins were extracted from normal controls and non-splenectomized β- thalassemia/HbE patients. In comparison with normal controls, the 21 proteins (spots no. 1, 2, 4-20) were up-regulated in non-splenectomized β- thalassemia/HbE patients and one protein (spot no. 3) was down-regulated.

Validation of Hsp70, IntegrinαIIb and PF4 protein by western blot analysis

To validate the 2D gel electrophoresis data, 3 differentially expressed proteins were validated by western blot analysis. The result showed that Hsp70, IntegrinαIIb and PF4 levels were increase in β- thalassemia/HbE patients when compared with normal controls. In particular, the splenectomized patients had higher expression levels than normal controls and non-splenectomized patients as shown in Figure 7.

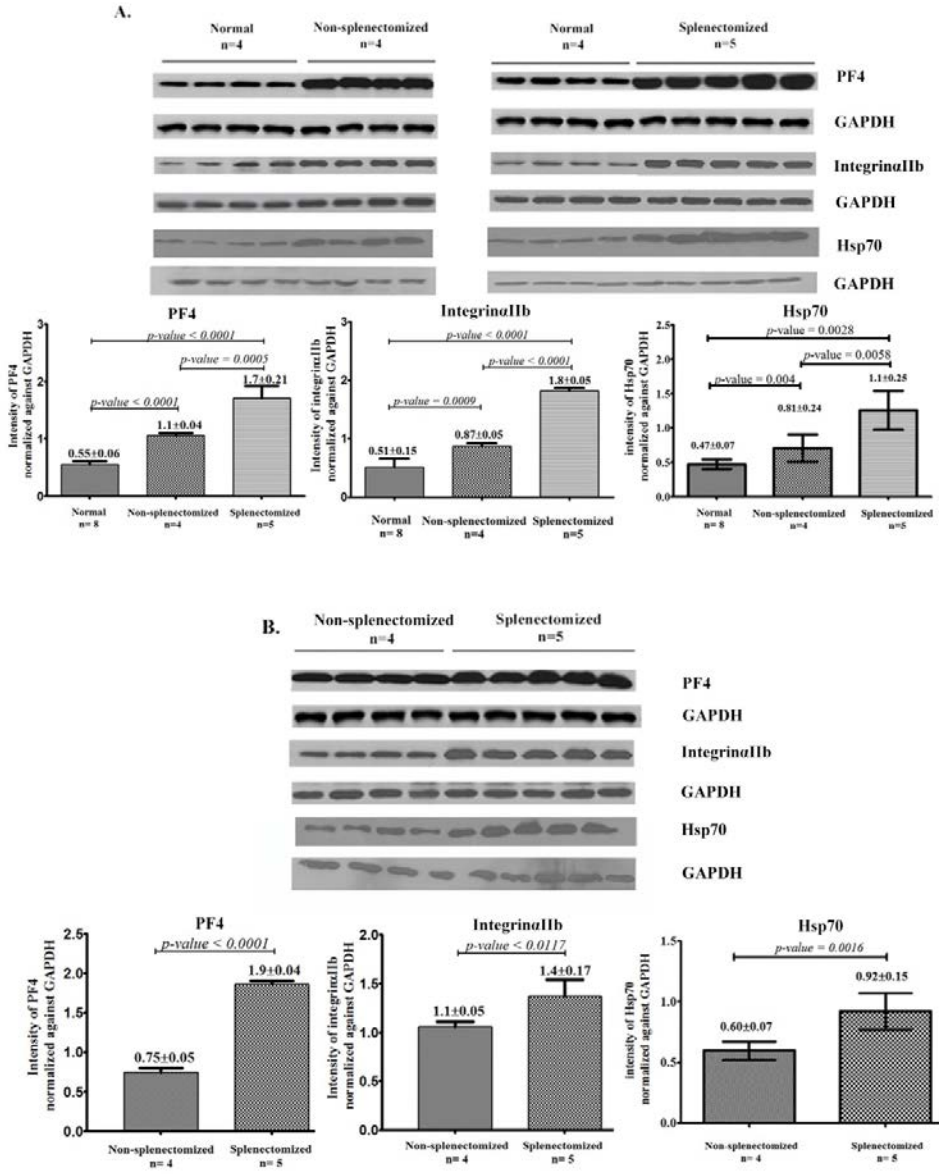

Figure 7 Expressions of PF4, IntegrinαIIb, HSP70 and GAPDH detected by Western blot analysis. Representative western blot analysis of PF4, IntegrinαIIb, and HSP70 against GAPDH in normal controls compared with β- thalassemia/HbE patients.

Output

International Journal Publications

1. Chanpeng P, Svasti S, Paiboonsukwong K, Smith DR, Leecharoenkiat K. Platelet proteome reveals specific proteins associated with platelet activation and the hypercoagulable state in beta-thalassmia/HbE patients. *Sci Rep.* 2019;9(1):6059. (Impact factor 4.122)

2. Kamonlak Leecharoenkiat, Pathrapol Lithanatudom, Wannapa Sornjai, Duncan R. Smith, Iron dysregulation in β-thalassemia, Asian Pacific Journal of Tropical Medicine 2016 Nov;9(11):1035-1043. (Impact Factor 1.062)

Other Related Activities

1. Oral presentation Title "Upregulation of Chaperones and Unfolded Protein Response in Hemoglobin H Constant Spring disease". The 5th TSH International Symposium "Red Cell Disorders: From Bench to Bedside", The Thai Society of Hematology, May 20-22, 2016, Bangkok, Thailand

SCIENTIFIC REPORTS

OPEN

Platelet proteome reveals specific proteins associated with platelet activation and the hypercoagulable state in β-thalassmia/HbE patients

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A hypercoagulable state leading to a high risk of a thrombotic event is one of the most common complications observed in β-thalassemia/HbE disease, particularly in patients who have undergone a splenectomy. However, the hypercoagulable state, as well as the molecular mechanism of this aspect of the pathogenesis of β-thalassemia/HbE, remains poorly understood. To address this issue, fifteen non-splenectomized β-thalassemia/HbE patients, 8 splenectomized β-thalassemia/HbE patients and 20 healthy volunteers were recruited to this study. Platelet activation and hypercoagulable parameters including levels of CD62P and prothrombin fragment 1 + 2 were analyzed by flow cytometry and ELISA, respectively. A proteomic analysis was conducted to compare the platelet proteome between patients and normal subjects, and the results were validated by western blot analysis. The β-thalassemia/HbE patients showed significantly higher levels of CD62P and prothrombin fragment 1 + 2 than normal subjects. The levels of platelet activation and hypercoagulation found in patients were strongly associated with splenectomy status. The platelet proteome analysis revealed 19 differential spots which were identified to be 19 platelet proteins, which included 10 cytoskeleton proteins, thrombin generation related proteins, and antioxidant enzymes. Our findings highlight markers of coagulation activation and molecular pathways known to be associated with the pathogenesis of platelet activation, the hypercoagulable state, and consequently with the thrombosis observed in β-thalassemia/HbE patients.

β thalassemia/HbE disease is the most common form of severe β thalassemia, responsible for approximately one-half of all severe β-thalassemia cases worldwide. The disease results from the co-inheritance of a β-thalassemia allele and hemoglobin E, the most prevalent structural β-globin variant in Southeast Asia. Patients that carry β-thalassemia/HbE show a clinical severity ranging from a severe transfusion-dependent thalassemia major to thalassemia intermediate[1]. The pathophysiology of β-thalassemia/HbE is mainly related to the accumulation of excess α-globin chains and its degradation products such as heme or hemin due to the imbalance of globin chain production. Ineffective erythropoiesis, peripheral hemolysis and iron overload are important factors responsible for the clinical manifestation of this disease[2,3].

Recently an increased risk for developing hypercoagulopathy, resulting in thrombotic events, has been observed in β-thalassemia/HbE patients[4]. The thrombosis mainly occurs in the venous system and manifests as a stroke, deep vein thrombosis or pulmonary embolism[5]. The hypercoagulopathy in patients with thalassemia has been attributed to several risk factors including abnormal RBCs, chronically activated platelets, enhanced platelet aggregation, iron overload, splenectomy, and increased prothrombin generation[6–9]. Platelets normally circulate in a non-activated state and play an important role in homeostasis and thrombin generation. The mechanism by which activated platelets induce thrombosis in β-thalassemia/HbE disease has not been fully elucidated. We sought to determine the platelet proteome related to the underlying mechanism of platelet activation and the

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Laboratory parameters	Healthy volunteers (n = 20)	β-thal/HbE patients (n = 23)	*p-value*	Non-splenectomy (n = 15)	Splenectomy (n = 8)	*p-value*
Age (mean ± SD)	28.0 ± 6.1	35.33 ± 9.3	0.3224	28.7 ± 6.2	34.0 ± 8.8	0.8750
Male female (n (%))	9 (45.0) 11 (55.0)	12 (52.2) 11 (47.8)	0.7626	7 (46.7) 8 (53.3)	5 (62.5) 3 (37.5)	0.6668
RBC × 10^6 cell/ul	4.3 ± 0.8	4.1 ± 1.0	0.3300	5 ± 1.0	3 ± 0.4	0.0036
Hb g/dL	14.0 ± 1.1	7.7 ± 1.2*	<0.0001	8.3 ± 1.1	6.5 ± 0.6	0.0005
Hct %	41.9 ± 2.4	26.2 ± 3.7*	<0.0001	27.6 ± 3.4	23.4 ± 2.2	0.0059
MCV fL	88.4 ± 4.2	66.4 ± 9.3*	<0.0001	62.9 ± 9.3	72.7 ± 5.3**	0.0138
RDW %	12.3 ± 0.4	22.1 ± 2.3*	<0.0001	22.2 ± 2.2	24.8 ± 1.1**	0.0067
NRBCs	0	133.0 ± 64.9*	<0.0001	4.5 ± 1.9	364.2 ± 295.4**	<0.0001
Reticulocyte count %	0.9 ± 0.1	5.4 ± 4.5*	<0.0001	2.0 ± 0.6	11.1 ± 1.0**	<0.0001
WBC Count × 10^3 cell/μL	5.9 ± 0.9	8.7 ± 4.3*	0.0072	6.1 ± 1.9	13.7 ± 2.7**	<0.0001
Platelet count × 10^3 cell/uL	289 ± 49	343.7 ± 231.2	0.3075	191 ± 65	630 ± 126**	<0.0001
Serum ferritin (ng/uL)	76.2 ± 37.8	979.1 ± 701.6*	<0.0001	512.2 ± 319.6	1757 ± 382.8**	<0.0001

Table 1. Comparison of lab finding between healthy volunteers and β-thalassemia/HbE Patients. Abbrevation: RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; RDW, Red blood cell distribution; WBC count, white blood cell count. Values represent mean ± SD. *Significant difference compared between healthy volunteers and β-thalassaemia/HbE patients at $p < 0.05$. **Significant difference compared between non-splenectomy and splenectomy β-thalassaemia/HbE patients at $p < 0.05$.

hypercoagulable state in the β-thalassemia/HbE disease by analyzing peripheral blood platelets isolated from healthy volunteers as compared to peripheral blood platelets from β-thalassemia/HbE patients with and without splenectomy. Our study provides novel insights to the underlying mechanism and pathophysiology of this disease.

Results

Clinical characteristic of β-thalassemia/HbE patients and healthy volunteers. Twenty-three β-thalassemia/HbE patients (15 non-splenectomized and 8 splenectomized patients) and 20 healthy volunteers were enrolled in this study. The hematologic data of all subjects are summarized in Table 1. The patients groups had lower Hb, Hct and mean corpuscular volume (MCV), and marked increases in the numbers of white blood cells, platelets, nucleated RBCs, and reticulocytes as compared to normal subjects. Comparison of hematologic parameters between β-thalassemia/HbE patients with and without splenectomy was also undertaken. As compared to the non- splenectomized group, the splenectomized patients showed lower Hb, Hct and MCV, but higher RDW and marked increases in the numbers of WBC, reticulocyte, nRBCs and platelets.

Increased platelet activation in β-thalassemia/HbE patients. By using flow cytometry, the isolated platelet populations were analyzed using plots of forward scatter (FSC-H) and side scatter (SSC-H). The population of isolated platelets in each subject group is found in the R2 region of the scatter plot (Fig. 1A). The platelet population in the R2 region was gated to analyze the percentage of contaminating RBC vesicles which are represented by the percentage of glycophorin A positive entities. The result showed that the platelet fraction of all subject groups contained contaminating RBC vesicles of less than 5%, including 0.6 ± 0.3% in samples from normal controls, 0.9 ± 0.5% in samples from non-splenectomized β-thalassemia/HbE patients and 3.9 ± 1.6% in samples from splenectomized β-thalassemia/HbE patients (Fig. 1B–D). These results indicate that the purity of isolated platelets is high, and they could be used for the other experiments. Entities at the R2 region were further gated and analyzed for the expression of a platelet specific marker (CD41a+) (Fig. 1E–G). The percentage of activated platelets (CD62P+) was gated from the R4 region (Fig. 1H–J). Samples from β-thalassemia/HbE patients showed a significantly higher percentage of activated platelets (CD62P+) as compared to samples from healthy volunteers and the samples from splenectomized patients showed a higher level than the samples from non-splenectomized patients.

Levels of prothrombin fragment 1 + 2 and its correlation with the platelet activation. The hypercoagulable state was evaluated by measuring the level of prothrombin fragment 1 + 2. Non-splenectomized β-thalassemia/HbE patients had threefold higher prothrombin fragment 1 + 2 levels than healthy volunteers. The highest levels of prothrombin fragment 1 + 2 were found in the splenectomized β-thalassemia/HbE patients (Fig. 2A), and the levels of prothrombin fragment 1 + 2 in the splenectomized patients were significantly higher than the levels found in the non-splenectomized patients. The correlation between levels of platelet activation and prothrombin fragment 1 + 2 among β-thalassemia/HbE patients was analyzed (Fig. 2B) and it was seen that the levels of platelet activation were closely correlated with the levels of prothrombin fragment 1 + 2 ($r = 0.6146$, $p = 0.0018$).

Comparative differential platelet proteome between healthy volunteers and β-thalassemia/HbE patients. The platelet proteome of 4 non-splenectomized β-thalassemia/HbE and 4 healthy volunteers were analyzed by 2D gel electrophoresis (Supplementary information). The spot expression levels were compared by

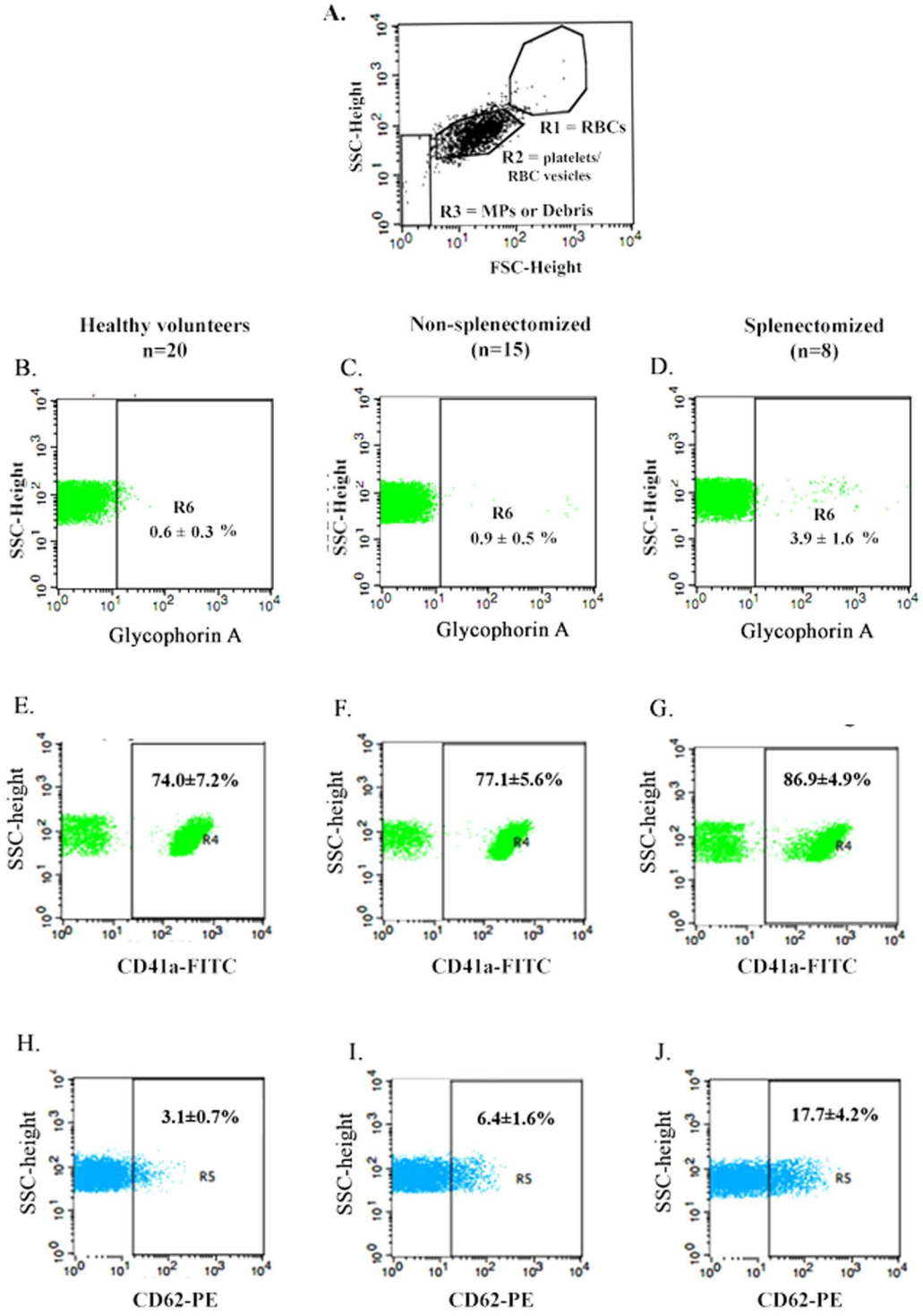

Figure 1. Purity of isolated platelets and level of platelet activation in β-thalassemia/HbE patients and healthy volunteers. After completing the isolation process, the isolated platelet fractions were analyzed by flow cytometry. Plotting between FSC and SSC displays the population of isolated platelets in the R2 region, while the R1 region is the population of red blood cells, and the R3 region is the fraction of microparticles or debris (**A**). The percentages of contaminating RBC vesicles in each subject group were analyzed by plotting between SSC and glycophorin A positives, which were gated from cells at the R2 region (**B**–**D**). The percentages of platelets markers were gated from R2 into R4 region (**E**–**G**). The levels of platelet activation for each subject groups were represented by the percentages of CD62P positive in the R5 region (**H**–**J**).

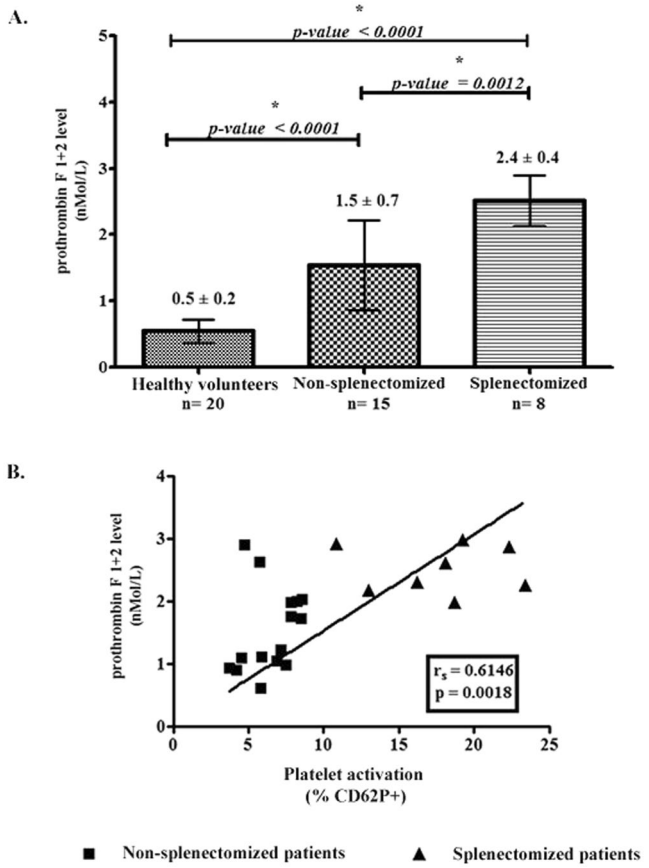

Figure 2. Level of prothrombin fragment 1 + 2 and it's the correlation with the platelet activation. The prothrombin fragment 1 + 2 levels of 8 splenectomized patients, 20 healthy volunteers and 15 non-splenectomized patients were detected by ELISA. The results of healthy volunteers, β-thalassemia/HbE patients and the reference value are shown in the columns with dashed lines represent mean ± SD (**A**). The spearman correlation coefficient was calculated between percentage of platelet activation (CD62P-positive) and levels of prothrombin fragment 1 + 2 (**B**).

analyzing the intensity, volume, and area of the protein spots. A total of 19 differentially regulated protein spots were identified, of which 18 spots were up-regulated and 1 spot was down-regulated in non-splenectomized β-thalassemia/HbE as compared to normal controls (Fig. 3). The spots were excised from the gels and subjected to tryptic in-gel digestion followed by mass spectroscopic analysis of the resultant peptides. A total of 19 proteins were identified as shown in Table 2. The down-regulated protein was identified as leukocyte elastase inhibitor. The largest groups of up-regulated proteins were cytoskeleton membrane proteins (F-actin -capping protein subunit beta, myosin light polypeptide 6, myosin regulatory light chain 12A, myosin-9, Rho GDP-dissociation inhibitor 2, transgelin-2, PDZ and LIM domain protein 1, and tropomyosin alpha-4 chain). The others upregulated proteins were a chaperone protein (heat shock protein 70 kDa protein), a fibrinogen receptor (integrin alpha IIb), an immune cell activation marker (beta-2-microglobulin), a globin protein (hemoglobin subunit beta) and platelet factor 4 (or chemokine (C-X-C motif) ligand 4 (CXCL4)).

Western blot analysis of integrin αIIb and platelets factor4. To validate the 2D proteomic analysis results, the levels of two differentially expressed proteins (platelet factor 4 and integrin αIIb) were measured by western blot analysis (Fig. 4). Platelet proteins were isolated from independent subjects (i.e. subjects whose samples were not used for the 2D analysis), including four non-splenectomized β-thalassemia/HbE patients, five splenectomized β-thalassemia/HbE patients and 8 healthy volunteers. The membranes were probed with antibodies directed to integrin αIIb and platelet factor 4 and normalized against GAPDH (Supplementary information). The results were consistent with 2D gel analysis in that the levels of integrin αIIb and platelet factor 4 were significantly increased in the non-splenectomized β-thalassemia/HbE patients when compared to healthy volunteers. Moreover, the splenectomized β-thalassemia/HbE had a higher level of those proteins as compared to the non-splenectomized patients.

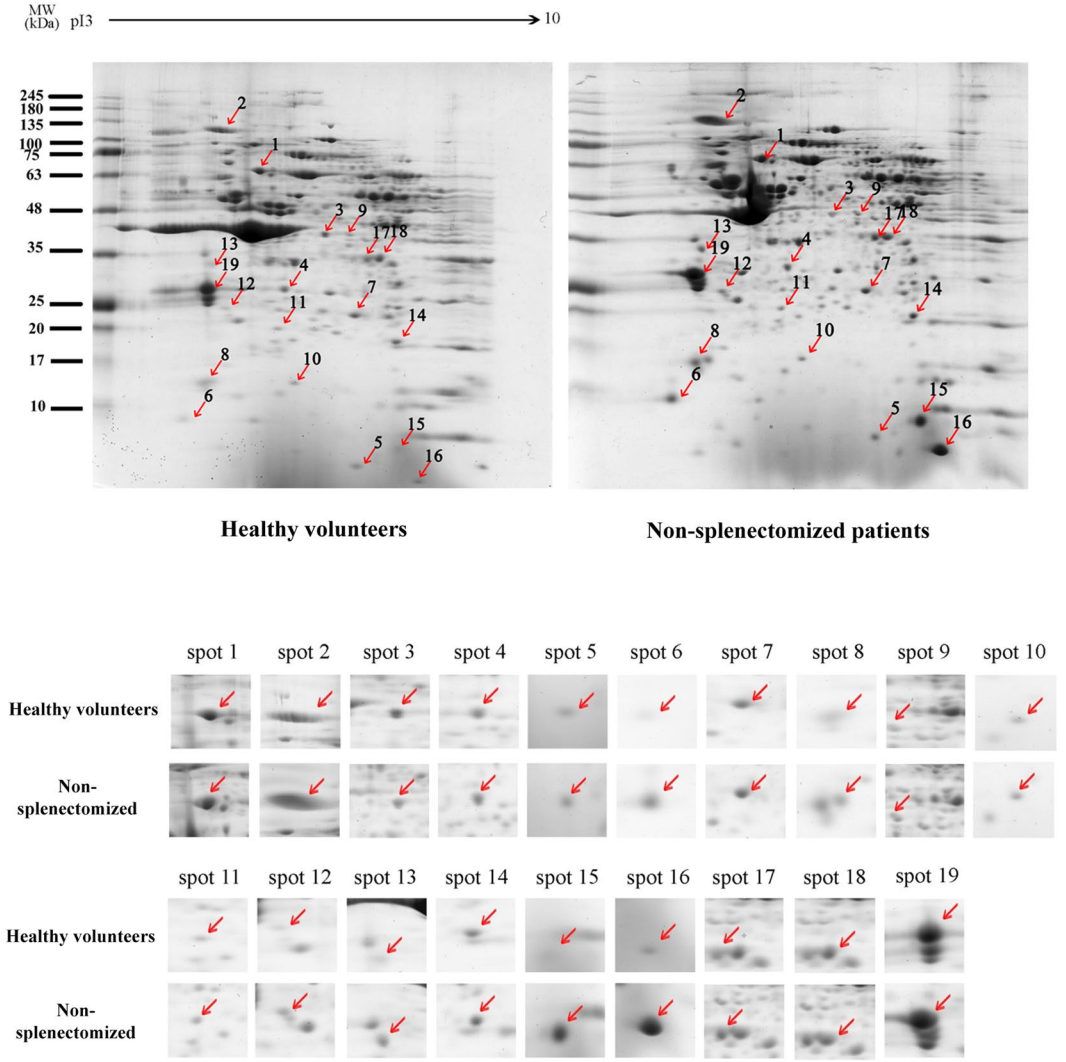

Figure 3. Platelet proteomes of non-splenectomized β-thalassemia/HbE patients and healthy volunteers. Platelet proteins compared between 4 non-splenectomized β- thalassemia/HbE patients and 4 healthy volunteers were subjected to 2D-electrophoresis following which gels were stained with Brilliant Blue Coomassie G250. The comparative 2D gels are representative of 4 replicates for each subject group. The resultant proteins spots were detected and analyzed by Image Master 2D-Platinum v7 software (Amersham Biosciences). The red arrows show 19 differential spots ($p < 0.05$) when comparing between the 2 sample groups. Eighteen of the protein spots were up-regulated and one protein spot was down-regulated (spot no. 3). The 19 protein spots that changed significantly were cropped from the same gel and enlarged.

Discussion

Hypercoagulability leading to a thromboembolic event is a significant factor influencing the morbidity and mortality of β-thalassemia patients. Several studies have shown higher platelet activation levels in β-thalassemia intermedia and splenectomized thalassemia patients than in healthy volunteers[8,10–14], and previous studies have indicated that the CD62P (P selectin) is a reliable marker for measuring platelet activation[15]. Confirming a previous report[16], we found significantly elevated platelet activation (CD41+/CD62P+) in non-splenectomized and splenectomized β-thalassemia/HbE patients. Increased levels of prothrombin fragment 1 + 2 (F1 + 2), which is an indicator of the hypercoagulable state, were also detected at higher levels in β-thalassemia/HbE patients as compared to healthy volunteers. While thrombosis is a known post-splenectomy consequence, our findings indicate a significant increase in platelet activation, hypercoagulation, serum ferritin as well as the up-regulation of platelet proteins in non-splenectomized patients, suggesting a closer monitoring of thrombosis in this group of patients is also needed. Moreover, it is noted that the expression of CD62P mediates the binding, rolling, and weak adhesion of platelets to leukocytes via P-selectin glycoprotein ligand 1 (PSGL-1)[17]. Previous study found that the levels of platelet-monocyte and platelet-neutrophil aggregation were elevated in β-thalassemia/HbE patients who had a hypercoagulation state. This result suggests that the platelet-leukocyte interaction might contribute to thrombin generation leading to the hypercoagulable state in β-thalassemia[18].

No	Protein name	MW(Da)	PI	Score	%Coverage	Fold change β/HbE vs healthy volunteers	Function
1	Heat Shock 70 kDa protein	70294	5.48	1033	25	1.41	-Chaperone protein
2	Integrin Alpha-IIb	114446	5.21	853	11	1.36	-Fibrinogen Receptor -Platelet activation and aggregation
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6	Myosin light polypeptide 6	17090	4.56	397	21	1.45	-Cytoskeleton
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9	Biliverdin reductase A	33692	6.06	203	26	1.09	-Heme synthesis
10	Actin -related protein 2/3 complex sub unit 5	16367	5.47	1147	47	1.32	-Cytoskeleton
11	Glutathione S-transferase P	23569	5.43	732	18	1.18	-Antioxidant enzyme
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13	Tropomyosin alpha- 1 chain	32746	4.69	134	13	1.04	-Cytoskeleton
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19	Tropomyosin alpha- 4 chain	28619	4.67	889	57	1.09	-Cytoskeleton

Table 2. Differential proteins compared between non-splenectomized β- thalassemia/HbE patients and healthy volunteers.

In order to identify platelet proteins associated with platelet activation or the hypercoagulable state in the β-thalassemia/HbE disease, a proteomic analysis was undertaken. The proteomic analysis showed 19 differentially expressed spots, which were identified by mass spectrometry as 19 different proteins. The proteomic analysis was not undertaken on platelets from splenectomized β-thalassemia/HbE patients as despite repeated attempts we were unable to obtain consistent isoelectric focusing, possibly as a result of high levels of coagulation factors in these samples. However, the proteins identified as differentially expressed between non-splenectomized β-thalassemia/HbE patients and healthy volunteers included a number of cytoskeletal proteins (F-actin -capping protein subunit beta, myosin light polypeptide 6, myosin regulatory light chain 12A, myosin-9, Rho GDP-dissociation inhibitor 2, transgelin-2, PDZ and LIM domain protein 1, and tropomyosin alpha-4 chain) as well as heat shock protein 70 kDa protein, integrin alpha IIb, beta-2-microglobulin, hemoglobin subunit beta, platelet factor 4 (or chemokine (C-X-C motif) and leukocyte elastase inhibitor. Of these proteins, all were shown to be up-regulated in β-thalassemia/HbE patients with the exception of leukocyte elastase inhibitor which was down-regulated.

One previous study has investigated the differential platelet proteome in the β-thalassemia/HbE disease[19]. That study identified 5 proteins (Hsp70, protein, disulfide-isomerase, eukaryotic translation initiation factor 5A-1, peroxiredoxin-2 and superoxide dismutase [Cu-Zn]). This study, and the study by Karmakar[19], only identified one protein in common, namely Hsp70. However, as reviewed elsewhere multiple studies determining the differences in proteomes between β-thalassemia/HbE patients and healthy volunteers rarely identify common proteins as a consequence of the usage of different samples sources (erythrocytes, platelet free plasma derived microparticles, plasma, etc) and different analysis methodologies, as extensively reviewed elsewhere[20]. In this regard we found higher serum ferritin levels in splenectomized patients than in non-splenectomized patients and healthy volunteers in contrast to the previous study[19]. Similarly, while the previous study[19] showed higher levels of protein C, which is a prothrombinase complex inhibitor, in splenectomized patients as compared to healthy volunteers and non splenctomized patients, our study showed increase activation of the prothrombinase complex as shown by the increased levels of prothrombin fragment 1 + 2 in the splenectomized patients.

As noted above, the majority of the differentially expressed proteins (10 out of 19) detected in this study were membrane skeleton proteins such as actin, myosin, tropomyosin and trangelin. These proteins are highly abundant in platelets and regulate contractile properties, indicating cytoskeleton re-organization during platelet activation[21]. In addition to the up-regulation of cytoskeletal proteins in platelets from β-thalassemia/HbE patients, we observed the up-regulation of integrin, a transmembrane receptor protein that acts as a bridge between the extracellular matrix and the cytoskeletal membrane that mediates signal transduction in activated platelets, resulting in platelet shape changes and platelet aggregation[22]. The up-regulation of integrin proteins is mediated by ADP, thromboxane A2 though a G protein –mediated signaling pathway which includes Rho GDP-dissociation inhibitor 2, which also seen as up-regulated in this study. This pathway activates a conformational change in the extracellular domains of proteins such as fibrinogen or von Willebrand factor, and fibrinogens act as bridges between

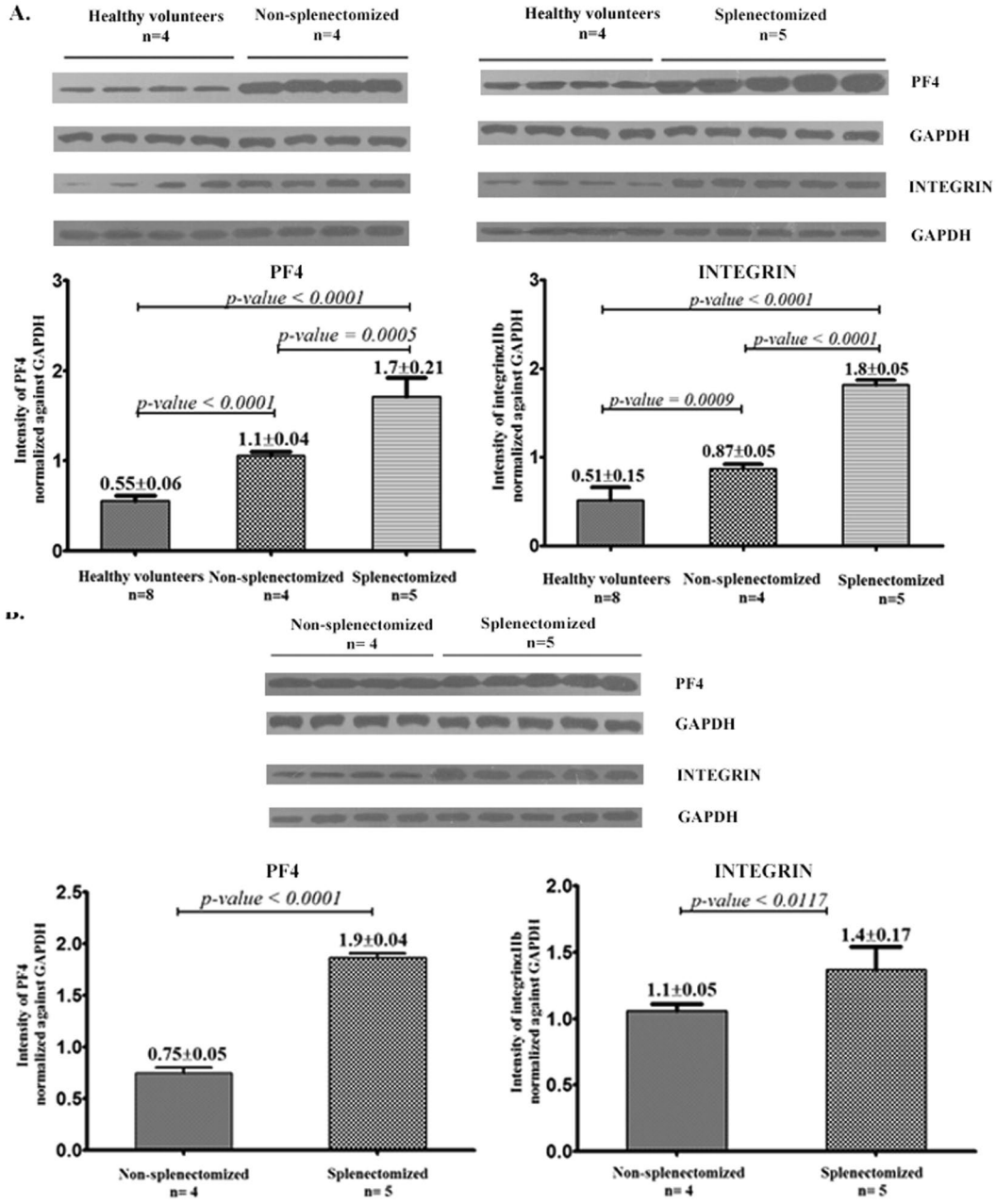

Figure 4. Expressions of PF4, Integrin αIIb and GAPDH detected by western blot analysis. Representative western blot analysis of PF4 and Integrin αIIb, normalized against GAPDH in healthy volunteers compared to the β- thalassemia/HbE patients are shown in figure (**A**). The comparison of protein expression between non-splenectomized β-thalassemia/HbE patients and splenectomized β- thalassemia/HbE patients are shown in figure (**B**). Band intensities for PF4 and Integrin αIIb were qualified and normalized against GAPDH and are shown as mean ± SD. The WB analysis for each protein compared between each subject group (healthy volunteer vs non-splenectomized patients, normal vs splenectomized patients and non-splenectomized vs splenectomized patients) was run in the same gel and transferred to the same nitrocellulose membrane. All membranes of the same comparing protein were exposed in the same X-ray film.

platelets to generate platelet aggregation[23]. Additionally, as noted above we also found the up-regulation of Hsp70, a specific chaperone that forms complexes with other co-chaperones in maintaining hemostasis and is associated with intracellular organization of signaling systems and platelet function[24], and expression of Hsp70 is an endogenous mechanism by which living cells adapt to stress[25]. Moreover, Hsp70 is associated with the inside-out activation of integrin-αIIbβ3 and the activation of platelet aggregation as well as granule secretion and platelet formation under conditions of physiological shear[26]. The consequences of signal transduction related to platelet integrin-αIIb and glycoprotein membrane changes are release of its granules including ADP (dense granules) and P-selectin and platelet factor 4 (PF4) (alpha-granules)[27] which were also up-regulated in this study.

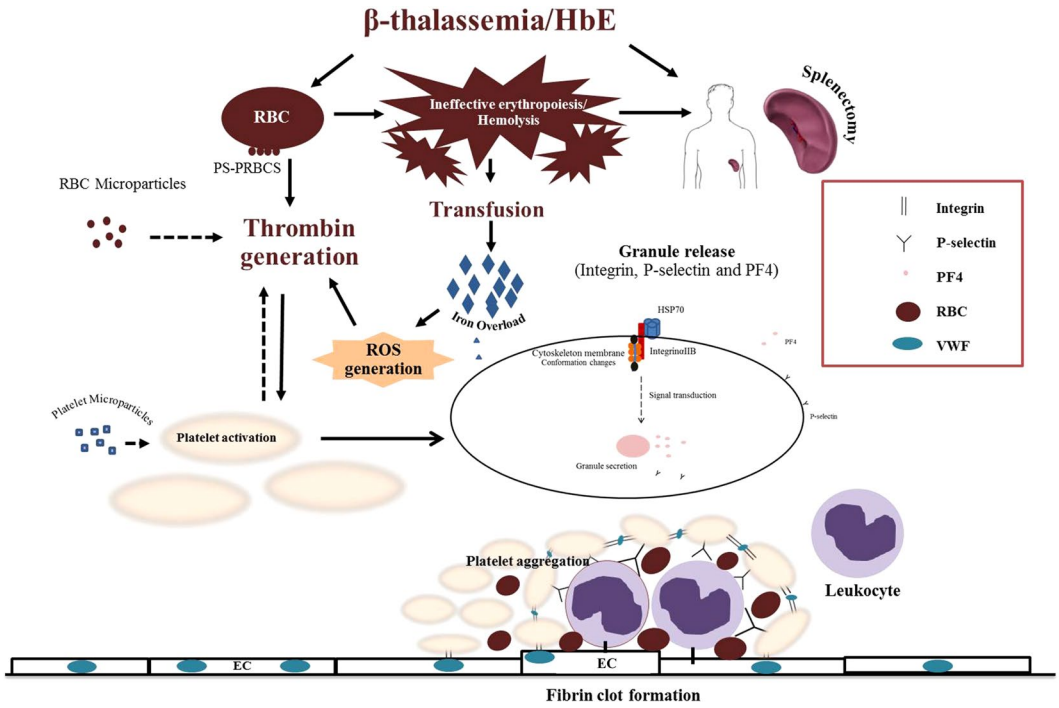

Figure 5. Schematic model illustrating a possible mechanism of hypercoagulable state in β-thalassemia/HbE. We proposed that the pathophysiology of β-thalassemia/HbE such as abnormal RBC and ineffective erythropoiesis induced PS- RBCs, hemolysis and splenectomy status in patients result in iron accumulation and increased ROS. These factors can indirectly and directly activate platelet alterations including platelet shape changes, secretion and aggregation leading to hypercoagulabilty in β-thalassemia/HbE patients.

Interestingly, the expression of platelet factor 4 (PF4) was a significantly higher in non-splenectomized and splenectomized patients. PF4 is a cytokine that is specifically released from alpha-granules of activated platelets[27]. PF4 plays a role in inflammation, atherosclerosis and thrombosis by neutralizing heparin anticoagulation to increased clot formation stability[28–30] and heparin-induced thrombocytopenia (HIT)[31]. The up-regulation of PF4 protein is a consequence of cytoskeleton, Hsp70 and integrin-αIIb protein changes, and the proteomic and western blot analyses showed very clearly increased levels in the β thalassemia/HbE patients. This result indicates that PF4 could be used as a potential diagnostic or predictive marker for the presence of thrombosis in β thalassemia/HbE patients. The others proteins identified as differentially expressed were related to heme synthesis (Biliverdin reductase A) and antioxidant enzymes (Peroxiredoxin 6, Glutathione S-transferase P) which may reflect an increase of free radical levels and consequent induction of antioxidant enzymes to protect against apoptosis[32].

Overall, our study confirmed the association of platelet activation and hypercoagulation, and identified a specific marker potentially predicting thrombosis which could improve early diagnosis and treatment intervention in β-thalassemia/HbE patients. We found significantly increased platelet activation, prothrombin fragment 1 + 2 levels and serum ferritin levels. Previous studies have shown a correlation of prothrombin fragment 1 + 2 levels and platelet activation with phosphatidylserine exposure on RBC[33,34]. Thus, platelet activation may be associated with several factors, including abnormal RBCs, RBC exposed phophotidylserine, micropartcles, hemolysis, iron overload and ROS resulting in chronic platelet activation and hypercoagulability in β-thalassemia/HbE patients (Fig. 5). It has been proposed that RBC hemolysis products (heme, hemin and iron) induce the generation of reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide[35] and that ROS are believed to affect platelet function and promote platelet activation both directly through the action of superoxide radicals, and indirectly through nitric oxide inhibition which contributes to platelet aggregation and thrombus formation[36]. Additionally, abnormal RBCs membranes lose phospholipid asymmetry, and expose phosphatydyl serine (PS) both on their membranes and on RBC derived microparticles which are shed into the blood circulation[37]. RBCs derived microparticles, a procoagulant, can enhance prothrombin complex formation leading to platelet activation which can additionally produced platelet derived microparticles that activate platelets[8,38,39]. When platelets are activated, the platelet skeleton membranes changes and they attach to the extracellular matrix and secrete chemokines and aggregate to others cells such leukocyte and endothelial cells leading to the formation of a plug[40]. Our results have shown the alteration of many platelet proteins leading to an understanding of pathways contributing to the hypercoagulable state in β-thalassemia/HbE patients. We suggest that possible preventive approaches for β-thalassemia/HbE patients with a hypercoagulable state or thrombosis include decreasing the level of RBCs exposing phosphatidylserine RBC, reducing ROS using antioxidants and inhibiting the coagulation pathway using anticoagulants.

Materials and Methods

Blood samples collection. This study subjects consisted of 23 β-thalassemia/HbE patients and 20 healthy volunteers. There was no significant difference in gender and age distribution between patients and healthy volunteers. Approval for the study was obtained from the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University with the certificate of approval no. 196/2016. Signed informed consent was obtained from all participants before blood collection. All methods were performed in accordance with the relevant guidelines and regulation of Chulalongkorn University. Complete blood cell counts were undertaken using a Sysmex XE 5000 hematology analyzer (Sysmex Corporation, Kobe, Japan). Diagnosis of β thalassemia/HbE was done by automated high performance liquid chromatography (HPLC) hemoglobin typing (VARIANT™, Biorad, Hercules. CA, USA) and reverse dot blot hybridization technique with the allele-specific oligonucleotide (ASO) probes followed standard published protocols[41]. Healthy volunteers were screened to be normal by complete blood cell count and hemoglobin analysis. The patients had not received a blood transfusion for one month, and had stopped medication for at least two weeks prior to blood sample collections. Serum ferritin levels were determined using a Ferritin Elisa kit (DiaMetra srl Unipersonel, Boldon, UK).

Platelet isolation method. Ten microliters of a citrate dextrose acid (ACD) blood samples were centrifuged twice at 200 g for 10 min at room temperature to collect platelet rich plasma (PRP). The platelets in the PRP fraction were inhibited by addition of 2 μM prostagladin E1 (Clayman Chemical, Michigan, USA) and incubation for 10 min at room temperature. After incubation the PRP fraction was centrifuged at 100 g for 10 min at room temperature to depleted red blood cell contamination. After centrifugation, the supernatants were collected in a new tube and centrifuged at 1500 g for 15 min to pellet the platelets. The platelet pellets were washed 3 times and re-suspended in 1 mL of 1x phosphate buffer saline (PBS) buffer, pH 7.4. One hundred microliters of platelet suspensions were kept to analyze platelet activation by flow cytometry. The remaining 900 μL of platelet suspensions were centrifuged at 13,000 rpm, 4 °C for 10 min to pellet the platelets which were kept for proteomic analysis. Levels of RBC vesicle contamination in the purified platelet fraction were measured by determining the levels of glycophorin A (GPA) positive fragments using an anti-human GPA antibody (BD Bioscience, Pharmagen, San Diago, CA) and analysis by flow cytometry.

Platelet activation analysis by Flow cytometry. After inhibition of *in vitro* platelet activation with prostagladin E1, 5 μL of the samples were stained with 1 μl of a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody against GPIIbIIIa (CD41a, BD Biosciences CA), 1 μl of a phycoerythrin (PE)-conjugated monoclonal antibody against P-selectin (CD62P, BD Biosciences, CA) and 1 μl of a allophycocyanin (APC)-conjugated monoclonal antibody against glycophorin A (GPA, Dakopatts Glostrup, Denmark). The mixtures were incubated for 15 min at room temperature. 150 μL of 1xPBS, pH 7.4 was added to the stained platelets which were immediately analyzed by flow cytometry (FACsClibur, BD Biosciences, San Jose, CA) as described elsewhere[42].

Measurement of prothrombin fragment 1 + 2 by ELISA. The presence of a hypercoagulation state was evaluated by measuring the level of prothrombin fragment 1 + 2 using a Human Prothrombin Fragment 1 + 2 (F1 + 2) ELISA Kit (MyBioSource, Inc., San Diego, CA, USA), according to the manufacturer protocol. The color intensity of the reaction was measured at density wavelength of 450 nm by an Infinite Pro M200 ELISA reader (TECAN, Switzerland). All the standard and test sample measurements were undertaken in duplicate. The level of prothrombin fragment 1 + 2 calculated from the standard curve using the Curve Expert software 1.4.

Two dimension electrophoresis (2D-PAGE). Platelet fractions were lysed with 150 μL of protein lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, and 1% human protease inhibitor cocktail. A total of 250 μg of soluble proteins containing 2% IPG buffer and 0.5% bromophenol blue were separated in the first dimension using an Immobiline dry strip, pH 3–10 NL, 7 cm. The protein solutions were rehydrated for 14 hours in reservoir slots of the re-swelling tray and isoelectric focusing was performed in a horizontal apparatus Ettan IPGphore3 (GE healthcare Life Sciences, UK). The IPG strips were equilibrated in equilibration buffer containing 100 mM DTT for 30 min and alkylated with 125 mg iodoacetamide (IAA) for 45 min. After equilibration, the proteins were separated in the second dimension using 12.5% polyacrylamide gels using an MiniVE vertical electrophoresis system (GE Healthcare Life Sciences, UK). The 2D gels were stained with 0.1% Coomassie Brilliant Blue G250 in 40% methanol for 24 hours and destained with deionized water for 6 hours to visualize the protein spots. After completion of the staining process, the gels were scanned under visible light at 600 μm/pixel resolution by an Image scanner III (GE Healthcare Life Sciences, UK). Spots were analyzed using Image master V.7 software (GE Healthcare Life Sciences, UK). Statistical analysis was performed by student *t*-test with a p value of <0.05 being considered significant.

Tryptic in-gel digestion and protein identification by LC/MS/MS. Differentially expressed protein spots were picked from the SDS-PAGE gels. The gel pieces were destained in a solution containing 50% ACN in 100 mL of 25 mM ammonium bicarbonate ($(NH_4)HCO_3$) and digested with trypsin for overnight at 37 °C in 25 mM ($(NH_4)HCO_3$). Peptides were extracted in 50 μL of 5% formic acid/50% ACN and then tubes were put into an ultrasonic bath for 15 min and samples subsequently were dried in a speed-vac. Peptide samples were dissolved in 98% H_2O, 2% ACN and 0.1% formic acid. The digested proteins were analyzed using an LC/MS/MS system consisting of a liquid chromatography part (Dionex Ultimate 3000, Thermo Scientific) in combination with an electro spray ionization (ESI)-ion trap mass spectrometer (amaZon SL, Bruker, Germany). The mass fingerprints were generated and searched against the SwissProt database protein (European Bioinformatics Institute, Cambridge, UK) database using the MASCOT search engine (Matrix Science, London, UK).

Western Blot analysis. A total of 30 µg of platelet proteins were separated by electrophoresis through 7% SDS-PAGE gels for the detection of integrin α2b protein and 12% SDS-PAGE gels for PF4 protein. After separation, the platelet proteins were transferred and blotted onto 0.2 µm nitrocellulose membranes (GE healthcare Life sciences, UK) using a Semi-Dry Blotter (Mini System, Cleaver Scientific, United Kingdom) and subsequently blocked with 5% non-fat milk in 1xTBS with 0.05% Tween (TBST) for 1 hour at room temperature. For analysis of the expression of PF4 the membranes were incubated with a 1:5000 dilution of a rabbit anti-PF4 polyclonal antibody (ab9561; Abcam, Cambridge, UK) in 5% BSA for 16 hrs. The membranes were washed 3 times with 1xTBS containing 0.05% Tween and incubated with a 1:10000 dilution of HRP-linked goat anti-rabbit IgG polyclonal antibody (Cell signaling Technology, Danvers, MA, USA). The expression of integrin α2b protein was detected by using a 1:1000 dilution of a rabbit integrin αIIb monoclonal antibody (D8V7H; Cell signaling Technology, Danvers, MA, USA) for 16 hrs followed by incubation with a 1:5000 dilution of HRP-linked goat anti-rabbit IgG polyclonal antibody (#7074 s; Cell signaling Technology, Danvers, MA, USA) for 1 hr. The protein levels were normalized against expression of GAPDH protein. Levels of GAPDH protein were determined using a 1:5000 dilution of a mouse anti-GAPDH monoclonal antibody (sc-32233; Santa Cruz Biotechnology Inc, Texas, USA) followed by a 1:10000 dilution of a HRP-linked rabbit anti-mouse IgG polyclonal antibody (Pierce, Rockford, IL, USA). All signals were visualized signal by adding the chemiluminescent ECL substrate (Boster Biological Technology, CA, USA) and exposure to X-ray film.

Statistical analysis. Statistical analysis of data was performed using an unpaired *t* test. Data are reported as mean ± standard deviation (SD) and the graphs were plotted using GraphPad Prism version 5.0. Spearman correlation coefficient (rs) was calculated to determine the correlation between platelet activation and prothrombin fragment 1 + 2. A *p*-value less than 0.05 was considered statistically significant.

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Author Contributions

P.C. and K.L. performed the research, analyzed the data and drafted the manuscript. K.L., S.S. and D.R.S. designed the research study. K.P. and P.C. were responsible for sample collections. K.L. and D.R.S. contributed to revising the manuscript and all authors approved the final version.

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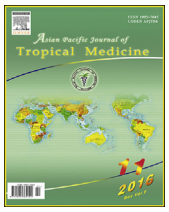

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Iron dysregulation in beta-thalassemia

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ABSTRACT

Iron deficiency anemia and iron overload conditions affect more than one billion people worldwide. Iron homeostasis involves the regulation of cells that export iron into the plasma and cells that utilize or store iron. The cellular iron balance in humans is primarily mediated by the hepcidin–ferroportin axis. Ferroportin is the sole cellular iron export protein, and its expression is regulated transcriptionally, post-transcriptionally and post-translationally. Hepcidin, a hormone produced by liver cells, post-translationally regulates ferroportin expression on iron exporting cells by binding with ferroportin and promoting its internalization by endocytosis and subsequent degradation by lysosomes. Dysregulation of iron homeostasis leading to iron deposition in vital organs is the main cause of death in beta-thalassemia patients. Beta-thalassemia patients show marked hepcidin suppression, ineffective erythropoiesis, anemia and iron overload. Beta-thalassemia is common in the Mediterranean region, Southeast Asia and the Indian subcontinent, and the focus of this review is to provide an update on the factors mediating hepcidin related iron dysregulation in beta-thalassemia disease. Understanding this process may pave the way for new treatments to ameliorate iron overloading and improve the long term prognosis of these patients.

1. Introduction

With the exception of a few species of bacteria, all living things need iron as an absolute requirement for viability. The ability of iron to act as both an electron donor and an electron acceptor makes it a critical component of many cellular oxidation/reduction reactions, and in addition iron is the substrate for heme, the critical component of hemoglobin, the essential oxygen carrying molecule of all vertebrates [1]. However, free iron is potentially extremely toxic to cells. Iron can donate electrons to oxygen resulting in the formation of the reactive superoxide radical (O^{2-}) or to hydrogen peroxide generating the hydroxyl (•OH) radical [1], and these molecules can oxidize biological macromolecules including lipids, proteins and DNA with extremely damaging consequences to the cell [2].

Humans contain approximately 3–4 g of iron in various forms [3]. Although iron is extremely plentiful in the environment, much of it is present in insoluble, non-bioavailable forms, and so humans have evolved to be highly efficient in conserving iron. Indeed, humans have no mechanism for excretion of excess iron under conditions of iron overload. Bioavailable iron in the diet serves mainly to replace iron lost from the body through processes such as the shedding of cells from the surface of the skin and lumen of the gut as part of the normal process of epithelial cell turnover. Additional loss of iron

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from the body may occur through minor bleeding events. In general it is believed that only some.

1–2 mg of iron (less than 0.1% of the total iron in a body) are lost from the body each day that require replacement though dietary sources [3].

The majority of the iron in humans is in the form of hemoglobin in red blood cells, and red blood cells combined contain between 2 g and 2.5 g of iron (out of 3–4 g in total). Red blood cells have a life span of some 120 days under normal conditions [2], after which they are degraded by macrophages and the iron returned to the plasma. Plasma contains some 2–3 mg of iron bound to a protein called transferrin, which is the primary molecule that transports iron for use in erythropoiesis in the bone marrow and by other iron requiring cells. Macrophages return some 20–25 mg of iron daily, ensuring a rapid turnover of iron in the plasma. When the binding capacity of serum transferrin is exceeded, iron starts to make complexes with other plasma proteins and molecules such as citrate. This iron is generally termed non-transferrin bound iron (NTBI) [4]. NTBI is easily taken up by hepatocytes and other parenchymal cells, and the intracellular accumulation of iron in these cells rapidly causes damage through oxidation reactions [2].

Within cells, iron is normally stored as the ferric (Fe^{3+} form) in association with a globular protein complex called ferritin. Ferritin is essentially a hollow sphere in which can sequester up to 4500 iron atoms. The ferritin complex consists of 24 subunits of heavy (H) and light (L) chains the exact composition of which can vary between tissues. The H chain has ferroxidase activity which converts Fe^{2+} to Fe^{3+} for storage inside the shell, while the L chain primarily stabilizes the structure and facilitates transport of iron ions to the inside of the structure [1]. This formation is the main storage system of iron (outside of the iron in hemoglobin). Under conditions of iron deficiency, iron is released from the complex to the plasma, while under conditions of mild iron excess the system can provide some buffering against the increased iron levels. In the average male, one gram of iron is held in storage mostly in hepatocytes and macrophages in the liver but also in spleen red pulp macrophages. Women of reproductive age tend to have significantly lower stored iron as a consequence of menstruation and childbearing [5].

As noted above, only a very small fraction of the total iron content is lost daily, and this is replaced through bioavailable iron sourced from the diet. Iron from the diet can be obtained from heme based sources (found in meat) and from non-heme iron sources (iron in cereals, vegetables, pulses etc). Iron absorption takes place in the gut duodenum and upper jejunum and occurs by transport across the apical membrane of enterocytes, which appears to occur through two independent pathways [6], one for heme iron and one for non-heme iron [3]. While absorption of non-heme iron is fairly well understood, the absorption of heme iron and ferritin iron is rather less well understood. Dietary non-heme iron is normally in the form of ferric iron (Fe^{3+}) which is reduced in the gut to the ferrous (Fe^{2+}) form by ferric reductase activity provided by duodenal cytochrome B and possibly Steap2 [3]. The ferrous iron is then transported across the apical (gut lumen) side of enterocytes by the ferrous iron transporter divalent metal ion transporter 1 (DMT-1), also known as Nramp-2 (natural resistance-associated macrophage protein) [7]. Some evidence suggests that heme iron may be taken up by receptor mediated endocytosis, although no high-affinity heme receptor has been identified to date [3]. There is some evidence that dietary ferritin is also taken up by endocytosis [8]. Once inside the enterocyte heme is broken down by heme oxygenase and dietary ferritin iron is released from ferritin. It is currently believed that iron from the various sources enters a common iron pool within the enterocyte. Some of the iron may be stored directly within the enterocyte as ferritin, while other iron will be released from the cell to end up bound to blood transferrin [8].

2. Intracellular iron trafficking and transportation

There is only one known cellular iron exporter, namely ferroportin [9–11]. This protein is found on the basolateral membrane of enterocytes as well as on other cells such as reticuloendothelial macrophages that export recycled iron, hepatocytes that release storage iron and on differentiating erythrocytes. Ferroportin exports iron in the ferrous (Fe^{2+}) form, but transferrin binds iron in the ferric (Fe^{3+}) form, so ferroxidases are believed to play a role in iron export. In intestinal enterocytes it is believed that hephaestin is the active ferroxidase, while in other cells this action is performed by the either circulating or GPI-linked multicopper ferroxidase ceruloplasmin [3,5]. Once bound to transferrin the iron is delivered to peripheral tissues by the transferrin–transferrin receptor system. After binding to the transferrin receptor, transferrin is internalized by receptor mediated endocytosis and upon acidification of the endosome iron is released from transferrin and converted to the ferrous form (Fe^{2+}) by the ferrireductase Steap family proteins [12,13] and transported across the membrane of the endosome into the cytoplasm by the action of the ferrous iron transporter divalent metal ion transporter 1 (DMT-1) protein [3,5].

Ferroportin has been shown to be regulated transcriptionally in enterocytes and macrophages [11,14] and to be translationally regulated by the iron responsive element (IRE) present in the 5′-UTR of the ferroportin mRNA through the action of iron regulatory proteins (IRP). The IRE–IRPs system is controlled by intracellular iron levels [9,11,15,16]. IRPs are activated during low iron condition under which they bind to the IRE of ferroportin mRNA resulting in translational suppression. Restrained ferroportin expression leads to reduced iron export, maintaining iron for cellular requirements. In addition, ferroportin is regulated at a post-translational step by the master iron homeostasis hormone, hepcidin. In erythroid precursor cells (and in enterocytes) a second mRNA encoding for ferroportin has been reported [17,18]. This mRNA is produced by the use of an alternate, upstream gene promoter and has an identical open reading frame in the mRNA, and as such the protein produced is identical. Critically, this second mRNA (termed FPN1B) does not contain an IRE in the 5′-UTR, and as such is not regulated by iron deficit [18]. It is currently believed that during erythropoiesis the relative expression of these two messages is coordinated to ensure that iron is exported from the cells during early differentiation, but kept in the cells during late differentiation when heme synthesis begins and iron demand is at its highest [18].

2.1. Iron regulation by hepcidin

The absorption of iron by enterocytes, the efflux of recycled iron from macrophages and the efflux of stored iron by

hepatocytes are all systemically controlled by the 25 amino acid peptide hormone hepcidin [19,20] which is produced predominantly in hepatocytes. Hepcidin is initially synthesized as an 84 amino acid preprohormone, before undergoing processing to generate a 60 amino acid prohormone and finally a 25 amino acid hormone [19]. The structure of the mature hormone is a compactly folded protein with 32% beta-sheet and 4 disulphide bonds [19]. Hepcidin regulates iron efflux by post-translationally negatively regulating ferroportin, the sole iron efflux channel. When iron is present in the plasma in excess hepcidin is secreted from the liver into the plasma. Hepcidin then directly binds to ferroportin expressed on the surface of iron storage cells, triggering endocytosis of both ferroportin and hepcidin which are subsequently degraded by lysosomes [21]. A reduction of ferroportin expression on the cell surface results in less intracellular iron being exported from storage cells, effectively locking iron inside the cell. This event reduces iron efflux into the plasma, returning iron to regular levels. The mechanism by which hepcidin regulates absorption of dietary iron is less clear as ferroportin is located on the basal surface of enterocytes cells, while absorption of dietary iron occurs on the apical surface. The mechanism by which a reduction in the basolateral ferroportin is communicated to the apical iron absorption mechanism remains unclear, although rising intracellular iron levels (as a consequence of reduced efflux) may play a role [5,21]. Mechanistically however it is known that hepcidin inhibits the uptake step of duodenal iron absorption but does not affect the proportion of iron transferred to the circulation [22].

2.2. Regulation of hepcidin expression

Hepcidin is primarily produced by hepatocytes [21], and hepcidin production is regulated by a number of factors, although it is believed that the primary stimuli are the level of iron in the plasma. Some studies have suggested that the two transferrin receptors (TfR1 and TfR2) together with the membrane protein hereditary hemochromatosis protein (*HFE*) serve to sense iron levels in the body and induce hepcidin expression, but the mechanism is incompletely understood [5]. A second pathway, the bone morphogenic protein (BMP) pathway, is probably activated in response to iron levels in intracellular iron stores, which results in increased expression of BMP6 which binds to a BMP receptor (BMPR) in association with a co-receptor hemojuvelin which activates the Smad signaling pathway resulting in increased hepcidin expression [5]. Recent evidence has shown that TMPRSS6, which encodes a hepatocyte-specific type II transmembrane serine protease, matriptase-2, cleaves hemojuvelin decreasing the BMP-SMAD signaling axis, and thus inhibiting hepcidin expression [23]. Inhibition of the expression of this inhibitory protein may provide an attractive pathway for increasing hepcidin expression in beta-thalassemia patients [24], although some drawbacks exist [25]. A further hepcidin stimuli is inflammation, and this pathway is mainly modulated through the inflammatory cytokine IL-6 which activates the JAK-STAT3 pathway leading to increased hepcidin expression in the liver [26,27].

To maintain iron homeostasis, the negative regulation of hepcidin expression is an important mechanism to ensure the availability of iron for biological activities. The erythropoietic cells of the bone marrow are the main consumers of iron provided by transferrin, and erythropoiesis is wholly dependent upon this source of iron. During erythropoiesis, erythroid cells secrete a factor or factors that suppress hepcidin expression in liver cells. This results in increased ferroportin activity and the transfer of iron from cellular stores to transferrin, thus supplying the demand for iron during erythropoiesis. The suppression of hepcidin also increases absorption of dietary iron [22]. The factor or factors secreted by erythroid cells remain to be clearly elucidated. Studies have implicated the growth and differentiation factor 15 (GDF15) which is known to be highly elevated in beta-thalassemia patients [28]. Other studies have suggested that the twisted gastrulation protein (TWSG1) may be the primary factor regulating hepcidin suppression in liver cells [29]. Moreover, erythroferrone has been recently identified and proposed as a candidate for suppressing hepcidin expression during erythropoiesis [30].

3. Thalassemia syndromes

Thalassemia syndromes are a group of inherited hematological disorders that constitute a major public health problems worldwide [31]. The term “thalassemia”, which has been used to describe autosomal recessive anemic disorders, is derived from the Greek word “thalassa” (the Mediterranean sea) and “haima” (blood) since it was first applied to the anemias frequently encountered in people around the Mediterranean sea, particularly in Italy and the Greek coast and nearby islands [32]. Thalassemia syndromes are a heterogeneous group of anemias which are caused by genetic defects in globin genes. Defect of one or more globin genes cause a partial reduction or total depletion of globin chains synthesis thereby leading to inadequate production of hemoglobin [33]. The major types of thalassemia are alpha- and beta-thalassemia which are classified according to the nature of the defective globin [33].

4. Beta-thalassemia

Beta-thalassemia is a heterogeneous group of disorders leading to decreased or absent beta-globin production. A genetic defect of one or two beta-globin genes, which are located on chromosome 11 (p15.5), is the cause of beta-thalassemia [34]. To date, more than 200 point mutations have been identified in beta-globin genes and the immediate flanking regions [35]. The expression of mutated beta-globin genes can result in reduced or absent beta-globin production, unlike the large gene deletions in alpha-globin gene, which solely result in loss of function [34]. According to these finding, beta-thalassemia can be phenotypically classified into 2 types; $beta^0$-thalassemia where no beta-globin chains are synthesized and $beta^+$-thalassemia where some beta-globin chains are synthesized [36]. In $beta^+$-thalassemia, there is a 5% to 30% reduction of beta-globin chains from normal levels [37]. The hallmark of beta-thalassemia is the reduced production or absence of Hb A ($alpha_2$ $beta_2$), reactivation of Hb F ($alpha_2gamma_2$), and importantly, accumulation of excess alpha-globin chains which appears to underlie the main physiopathology of the disease. Patients with the most severe form of beta-thalassemia ($beta^0$/$beta^0$) develop serious microcytic anemia due to severe hemolysis and impaired production of new RBCs [38]. Bone deformity

as a result of erythroid hyperplasia and enlargement of liver and spleen are also observed [35]. In heterozygous beta-thalassemia (beta/beta^0, beta/beta$^+$) or beta-thalassemia traits, Hb A formation is substantial due to the output from the remaining intact beta-globin gene, thus resulting in a nearly asymptomatic presentation with mild hypochromia with microcytosis but with relatively little evidence of anemia, hemolysis, or impaired erythropoiesis.

The most common subgroup of beta-thalassemia is beta-thalassemia/Hb E which accounts for almost 50% of the patients with severe beta-thalassemia worldwide [38]. This compound heterozygote is very common in many regions where Hb E is predominant. Hemoglobin E (Hb E) is the structural hemoglobin variant which is the most common in Southeast Asia including Thailand (10–50% of the population) [39,40]. The betaE-globin gene produces only small amounts of betaE-globin chains, which is similar to some mutations causing beta$^+$-thalassemia and therefore Hb E trait resembles a very mild beta$^+$-thalassemia trait while Hb E homozygotes exhibit more microcytosis but are still asymptomatic [41]. The severity of beta-thalassemia/Hb E generally depends on the co-inheritance of alpha-globin hemoglobinopathies as well as the level of Hb F. Although patients with beta$^+$-thalassemia/Hb E develop a mild anemia with only a few clinical abnormalities, an extraordinarily wide clinical spectrum, ranging from a moderate to a severe form of anemia resembling homozygous beta^0-thalassemia are observed in beta^0-thalassemia/Hb E patients [40,42–44].

4.1. Molecular pathogenesis of beta-thalassemia

The main pathophysiology of beta-thalassemia is caused by the unbalanced production of alpha-globin and βετα-globin chains where alpha-globin chains appear to be in excess [35]. Unlike beta-globin chains, alpha-globin chains are unable to form stable tetramers thus free excess alpha-globin chains tend to form insoluble aggregates which precipitate within the developing erythroid cell. This results in the induction of apoptosis in the developing erythroid precursor at the polychromatophilic normoblast stage in a process termed ineffective erythropoiesis [45]. In the small percentage of erythroid cells that progress to maturation, the accumulation of free alpha-globin chains efficiently generates ROS and oxidative stress, resulting in RBC membrane damage and subsequently increased hemolysis [46]. In normal RBCs hemoglobin is reversibly oxidized to methemoglobin, with cytochrome b5 reductase mediating the reduction back to hemoglobin. However, free globin chains (both alpha and beta) are susceptible to oxidation to hemichromes which can become irreversibly modified [47], allowing the hemichrome iron to generate reactive oxygen species [48]. A large part of the difference in pathology between αλπηα- and βετα-thalassemia arises from the fact that the excess βετα-chains present in αλπηα-thalassemia can form a soluble tetramer (hemoglobin H, HbH) while the excess αλπηα-globin chains present in beta-thalassemia cannot, resulting in the deposition of insoluble aggregates in the RBC membranes. In beta-thalassemia therefore, the combination of ineffective erythropoiesis of the developing erythroid precursor cells and increased hemolysis of the mature RBC are the main causes of anemia in these patients. The anemia in these patients leads to a feedback loop that results in increased expansion of erythroid progenitors and accelerated erythroid differentiation [49]. The markedly increased erythropoiesis in beta-thalassemia has been reported in many studies either by prediction, based on ferrokinetic studies [50], by differential counting from bone marrow aspirates [51], or by *in vitro* observation of CD34 hematopoietic progenitor culture [45,52–54]. The marked expansion of the erythroid mass is a well documented feature of intermediate and severe beta-thalassemia cases which results in the generation of more distressing features such as organ enlargement and particularly bone deformity and fragility [42,55,56]. Ultrastructural studies using electron microscopy have shown that the precipitated αλπηα-globin chains are in both the cytoplasm and the nucleus [57] and begin to be present predominately in polychromatic erythroblasts [58–60]. Moreover, abnormal erythroid nuclei showing a partial loss of nuclear membrane and presence of intranuclear aggregates of αλπηα-globin chains have also been observed in bone marrow erythroblasts of homozygous βετα-thalassemia patients [58]. These findings led to investigations of intramedullary death which showed later that programmed cell death or apoptosis clearly occurred in erythroid precursors of beta-thalassemia major as detected by DNA ladder formation [61] and the outer externalization of phosphatidylserine (PS) to the membrane leaflet [62]. Additionally, *in vitro* studies have also demonstrated that apoptosis primarily occurs at the polychromatophilic normoblast stage [45], the intermediate stage of erythroid precursor differentiation where the αλπηα-globin chain aggregates appear to present [58]. Previous studies have demonstrated that heat shock protein 70 (HSP70) interacts directly with excess free αλπηα-globin chains and is sequestered in the cytoplasm [63]. This prevents HSP70 from performing its normal physiological role of protecting GATA-binding factor 1 (GATA1) from proteolytic cleavage resulting in premature degradation of GATA1 and maturation arrest and apoptosis of polychromatic normoblasts [64]. The marked degree of anemia, due to ineffective erythropoiesis, combined with a considerable tissue hypoxia promote erythropoietin (EPO) production, which has been shown in several studies to be increased in beta-thalassemia/Hb E patients as compared to normal controls [52,65–67]. Increased levels of EPO are believed to be the main factor driving the expansion of the erythroid mass. Extensive erythropoiesis induces a large erythropoietic mass which can be found in the bone marrow, liver and spleen, as well as at extramedullary sites.

4.2. Iron overload in beta-thalassemia

Severe cases of beta-thalassemia require regular blood transfusion to reduce the chronic anemia. Multiple blood transfusions, increased hemolysis of red blood cells and increased gastrointestinal iron absorption (Figure 1) lead to iron overload [68], and cardiomyopathy as a consequence of iron overloading is the most common cause of death in transfusion-dependent thalassemia patients [69]. The human body loses only 1–2 mg iron per day, while a unit of transfused red blood cells contains approximately 200 mg of iron [38]. A patient who receives 25 units of blood per year, accumulates 5 g of iron each year in the absence of any iron chelation therapy. Excess iron is extremely toxic to all cells of the body and can cause serious and irreversible organic damage, such as cirrhosis, diabetes, heart disease, and hypogonadism which lead to significant morbidity and mortality if untreated [68]. The iron overload on the body can be estimated by means of serum

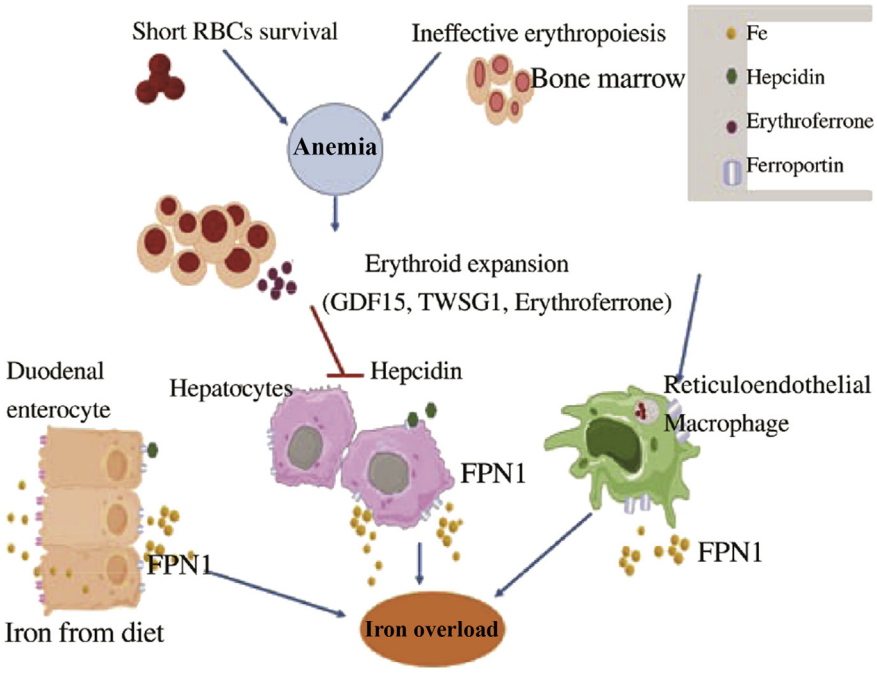

Figure 1. Proposed mechanism of iron dysregulation in beta-thalassemia disease.
Beta-thalassemia patients experience anemia mainly from ineffective erythropoiesis and shortened red blood cell (RBC) survival. The anemia induces erythropoietin (EPO) production leading to enhanced erythropoiesis. The dramatically increased erythroid expansion activates secretion of erythroid factors including growth differentiation factor 15 (GDF15), twisted-gastrulation 1 (TWSG1) and erythroferrone (ERFE). Excessive erythroid factors suppress hepcidin expression in hepatocytes resulting in increased iron absorption from duodenal enterocytes, the release of iron from the liver and the reticuloendothelial system, culminating in iron overload.

ferritin, hepatic iron concentration, urinary iron excretion and TIBC levels [70]. Threshold values for iron toxicity are a liver iron concentration exceeding 440 mmol/g dry weight, serum ferritin >2500 ng/mL, urinary iron excretion >20 mg/day, and transferrin saturation >75% [71]. The estimation of hepatic iron concentration by magnetic resonance imaging (MRI) is the most commonly employed test to evaluate iron overload in thalassemia major [72]. Increased iron overload has also been reported in patients with non-transfusion dependent thalassemia (NTDT) [73]. beta-thalassemia carriers and patients who have a histidine to aspartic acid (H63D) mutation at codon 63 of the *HFE* gene show iron overloading, suggesting that the H63D mutation may have a modulating effect on iron absorption [74,75].

4.2.1. Pathophysiology of iron overload in beta-thalassemia

The excess iron in beta-thalassemia patients saturates the ability of the transferrin iron transport system, leading to non-transferrin bound iron (NTBI) and labile plasma iron (LPI) starting to circulate in plasma and subsequently becoming deposited inside susceptible cells [71]. Rather than using the transferrin receptor, NTBI enters cells by other cellular channels including L-type voltage-dependent Ca^{2+} channel (LVDCC), a promiscuous divalent cation transporter [76] and Zip14, a member of the SLC39A zinc transporter family [77]. Long-term uptake and accumulation of NTBI and LIP, its redox active component, leads to increased levels of storage iron and labile cellular iron [78]. Tissues susceptible to iron accumulation by this mechanism include the liver, endocrine system and myocardium [79]. When the magnitude of the cellular labile iron pool exceeds the capacity of the cell to synthesize new ferritin molecules, a critical concentration is reached that can generate reactive oxygen species (ROS). ROS produced by the metabolism of NTBI plays a central role in inducing cellular dysfunction, apoptosis, and necrosis [80]. A variety of ROS, most notably hydroxyl radicals, increase lipid peroxidation and organelle damage, leading to cell death and fibrogenesis mediated by transforming growth factor beta1 (TGF-beta1) [81]. An underappreciated effect of iron overload is increased infection risk that is a high cause of mortality in beta-thalassemia patients [82]. Oxidative stress is a major inducer of autophagy, which is important in the removal of oxidized proteins and damaged mitochondria. The increased activation of autophagy has been reported in beta-thalassemia/Hb E erythroblasts as compared to normal control erythroblasts [83], suggesting that high levels of autophagy in beta-thalassemia/Hb E erythroblasts might be induced by ROS that contribute to the increased levels of apoptosis that lead to ineffective erythropoiesis in beta-thalassemia/Hb E erythroblasts [83].

Recently, dysregulation of ferroportin *mRNA* has been reported in beta-thalassemia/Hb E. While erythroblasts from normal controls show increased expression of ferroportin expression during differentiation under iron overload conditions, erythroblasts from beta-thalassemia/Hb E patients show no increase in ferroportin expression under the same growth conditions [84]. Thus the ability of these critical erythroid cells to export excess iron is curtailed, adding to the direct biological consequences of iron overload.

Iron overload can also contribute to ineffective erythropoiesis to a varying extent depending on the disorder (Figure 1). It has been suggested that the production of growth differentiation factor 15 (GDF15) [28,85] and possibly other proteins, such as twisted-gastrulation 1 (TWSG1) [29], contributes to the inhibition of hepcidin synthesis and thus promotes iron

absorption in beta-thalassemia patients. Kautz and colleagues suggested that, upon increased erythropoiesis, bone marrow and spleen erythroblasts increasingly produce erythroferrone, which, upon secretion into the circulation, directly acts on the liver to inhibit hepcidin production and mediates increased iron mobilization and absorption during periods of erythropoietic stress [30].

4.3. Hepcidin expression in beta-thalassemia

Deficits in hepcidin expression in relation to thalassemia were first reported in a mouse model system (C57BI/6 $Hbb^{th3/+}$) of severe anemia [86], and since then hepcidin levels in beta-thalassemia/Hb E disease, beta-thalassemia trait and Hb E trait carriers have been reported [87]. Levels of hepcidin in beta-thalassemia patients have been shown to be extremely low [88–91] and serum from beta-thalassemia patients has been shown to suppress hepcidin expression in liver cells [28]. This will lead to continual, effectively unregulated absorption of dietary iron, leading to overloading. Liver hepcidin mRNA expression in patients with thalassemia major and thalassemia intermedia is inversely correlated with soluble transferrin receptor (sTfR) and erythropoietin (EPO), but not with iron stores [92]. The study proposed that hepcidin suppression in beta-thalassemia/Hb disease is associated with iron loading, saturation of iron binding proteins, and consequently, organ damage as indicated by an inverse association between hepcidin and NTBI across all patients, as well as correlation of NTBI and ferritin or LIC, and correlation of iron loading with ALT, an enzymatic marker of hepatic damage [87]. While moderate suppression of hepcidin with enhanced iron absorption was also observed in beta-thalassemia carriers; this was not the case with Hb E trait carriers [87,93]. The coinheritance of alpha-thalassemia results in a reduction in erythropoiesis and ameliorates hepcidin suppression [87]. Less severe forms of ineffective erythropoiesis, as observed in alpha-thalassemia, may cause late-onset and milder iron overload.

4.4. Hepcidin in the pathogenesis of beta-thalassemia

Anemia, tissue hypoxia and increased EPO production observed in beta-thalassemia promote the suppression of hepcidin and increase iron absorption in response to the demand for iron by erythroblasts [94]. Several hepcidin inhibitors released from erythroblasts during the process of differentiation have been proposed to regulate hepcidin expression in beta-thalassemia. The cytokine members of the TGF-beta family, namely growth differentiation factor 15 (GDF15) was shown to be up-regulated in serum from thalassemia patients and can suppress hepcidin expression in hepatoma cells or in isolated human hepatocytes [28,95]. Serum levels of this cytokine are strikingly elevated in patients with homozygous beta-thalassemia, while intermediate levels are found in carriers of alpha-thalassemia and in beta-thalassemia trait carriers. In contrast, sickle cell patients whose anemia is related to chronic hemolysis rather than ineffective erythropoiesis, show no or only modest GDF15 elevation [28]. TWSG1 is a second erythroid factor that has been identified as a hepcidin regulator. Levels of this protein are increased in the bone marrow, spleen and liver of thalassemic mice [29]. However, the level of TWSG1 in the serum of thalassemia patients remains to be reported. Hepcidin inhibition by the liver serine protease TMPRSS6 has also been shown [23,96]. More recently, the hormone erythroferrone (ERFE) has been identified as a new erythroid regulator of hepcidin synthesis [30,97]. In mouse models of intermediate thalassemia, bone marrow ERFE expression is increased in response to erythropoietin and mediates hepcidin suppression during stress erythropoiesis. ERFE-deficient mice fail to suppress hepcidin rapidly after hemorrhage and mice exhibit a delay in recovery from blood loss [97]. However, the molecular mechanisms underlying the suppression of hepcidin by these erythroid factors, as well as the interplay between the factors remain to be clarified.

4.5. Therapeutic targeting of hepcidin in beta-thalassemia

The standard treatment of severe beta-thalassemia is currently based on blood transfusions, iron chelation and splenectomy, allowing an increased survival and amelioration of the patients' quality of life [98]. A cure for inherited beta-thalassemia can be achieved by allogeneic hematopoietic stem cell transplantation, but the need to control transplant-related complications and the requirement for matched donors make this option available to only some patients [99], and as many as 60% of patients lack a suitable donor [99]. Alternative treatments, such as gene therapy or the induction of fetal hemoglobin (Hb F) are promising [100], but have yet to make it to the bedside. As iron overload is the most important complication for the patients with blood transfusion, iron chelation is essential to control iron overload and its toxicity [101] and effective management of iron overload in thalassemia requires monitoring both for iron toxicity and the effects of excessive chelation. Recently however, improved knowledge of the relationships between iron overload and hepcidin has led to the development of novel approaches that target the pathophysiology of the disease with the aim of reducing iron overload and, at the same time, of alleviating ineffective erythropoiesis. Hepcidin levels are low in thalassemia patients with concomitant pathophysiological consequences, and the restoration of hepcidin to normal levels is an attractive novel therapeutic strategy. Studies in a mouse model of beta-thalassemia have shown that increasing hepcidin reduces iron bioavailable to erythroblasts, resulting in decreased heme synthesis and improved erythroid precursor and reticulocyte survival [102]. Similarly small synthetic peptides (minihepcidins) can decrease serum iron, prevent iron overload and promote iron redistribution in hepcidin-deficient mice [103]. In another approach, administration of BMP6, the natural ligand of the BMP receptor involved in hepcidin regulation, was shown to activate the hepcidin transcription regulated pathway and to correct the high iron saturation and iron maldistribution in a *HFE* model of hereditary hemochromatosis [104]. Transgenic inactivation of the membrane protease TMPRSS6 in *HFE* mice increased hepcidin expression and reversed their iron overload phenotype, suggesting that the administration of a specific inhibitor of the enzymatic activity of TMPRSS6 could be used to treat iron overload [105,106]. An RNAi therapeutic targeting Tmprss6 has been shown to decrease iron overload with diminished hepcidin expression and may have efficacy in modifying disease-associated morbidities of thalassemia [24]. A combination therapy of RNAi against Tmprss6 together with

administration of the iron chelator deferiprone has been reported to result in a significant reduction of liver iron content and improved erythropoietic efficiency in thalassemic mice [107,108].

5. Conclusions

While the genetic lesions engendering beta-thalassemia are well characterized, how these lesions lead to the full pathological spectrum of the disease remain less well understood. While deposition of unpaired αλπηα-globin chains during erythropoiesis is a major event, it is clear that dysregulation of iron homeostasis in both transfusion dependent and transfusion independent beta-thalassemia patients is a dominant physiological effect. Understanding hepcidin expression and regulation in the context of the beta-thalassemia patient is vital to developing rational therapeutic interventions to provide safe, effective and lifelong treatments.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Upregulation of Chaperones and Unfolded Protein Response in Hemoglobin H-Constant Spring Disease

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INTRODUCTION

HbH-CS results from the co-inheritance of the α^{SEA} deletion with HbCS which is characterized by a point mutation of the termination codon of the α2 globin gene. Accumulation of excess β globin chains and HbCS in this disease leading to RBC membrane alterations as oxidation of phospholipids, modification of cytoskeletal proteins and their interactions, reduced membrane-associated ATPase activities, and enhanced permeability of cations. This study sought to determine whether there were alterations in the RBC pathways of HbH-CS other than these abnormalities. Proteomic analysis of RBC proteins comparing between normal and HbH-CS patients was analyzed.

EXPERIMENT DESIGN

Packed red cells proteins were separated from 8 HbH-CS and 8 normal subjects. The protein profiling was analyzed using two-dimensional gel electrophoresis. The differential protein spots were identified using LC/MS/MS analysis and validated by western blot analysis.

RESULTS

A total of 20 differentially expressed spots were detected of which 10 spots were up regulated and 10 were down regulated. Down regulation of 3 RBC membrane proteins (Ankyrin, Topomyosin and Integrin) and up regulation 3 of chaperone or unfolded protein response proteins (HSP70, GRP78 and Calreticulin) in HbH-CS was detected.

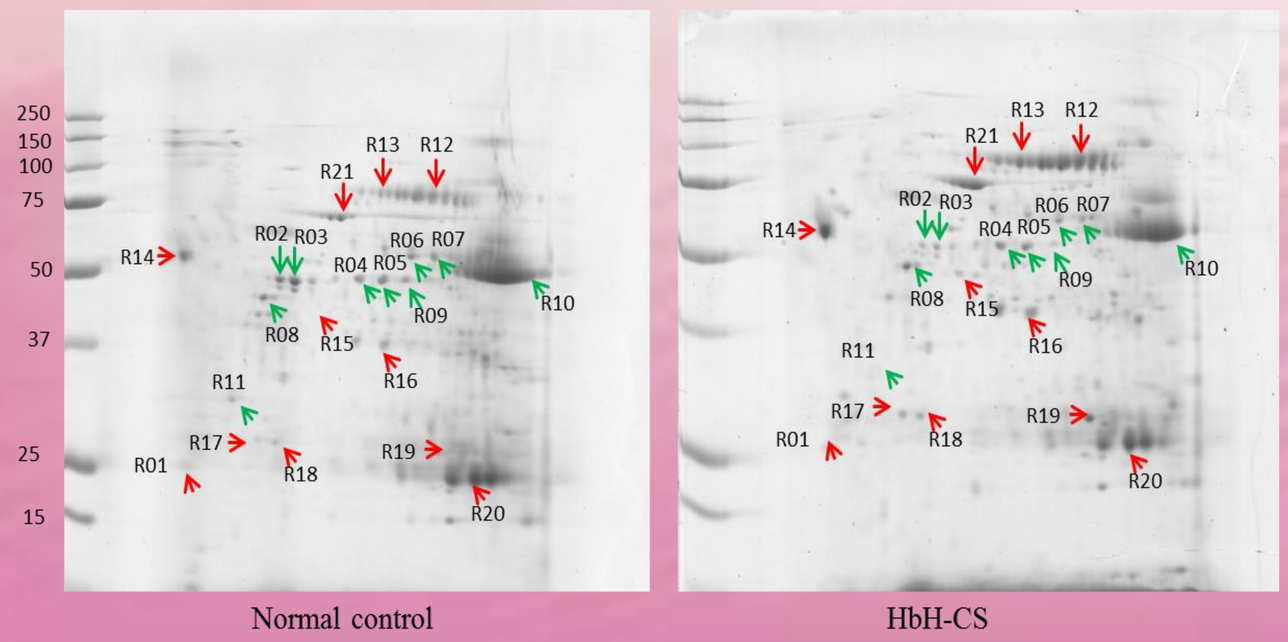

Figure 1. Representative 2DE of RBC proteins. Differential expression of RBC proteins comparing between normals and HbH-CS patients. A total of 20 significantly different protein spots with 10 up- regulated proteins (red arrow) and 10 down regulated proteins (green arrow) were detected.

Table 1. List of 20 differential spots with 19 differential RBC proteins compared between normals and HbH-CS patients

No	Name of protein	Score	% coverage	Normal/ HbH	Cellular localization
R01	Immunoglobulin J chain	99	3	Up regulation	Blood microparticle
R02	Fibrinogen gamma chain	376	19	Down regulation	Cell surface
R03	Fibrinogen gamma chain	438	16	Down regulation	Cell surface
R04	Integrin alpha-E	553	37	Down regulation	Cell membrane
R05	Heme oxygenase 2	58	16	Down regulation	Cytoplasm
R06, 07	Beta-2-glycoprotein	42	16	Down regulation	Cell surface
R08	78 kDa glucose-regulated protein	696	73	Up regulation	Endoplasmic reticulum membrane
R09	Fibrinogen beta chain	172	13	Down regulation	Cell surface
R10	Ankyrin-1	172	13	Down regulation	Cytoplasm, cytoskeleton
R11	Tropomyosin alpha-4 chain	102	13	Down regulation	Cytoplasm, cytoskeleton
R12	Ig mu chain C region	325	34	Up regulation	Blood microparticle
R13	C4b-binding protein alpha chain	46	10	Up regulation	Plasma membrane
R14	Calreticulin	141	29	Up regulation	cell membrane
R15	Proliferation-associated protein	115	24	Up regulation	Cytoplasm
R16	Heat shock 70 kDa protein	363	20	Up regulation	Cytoplasm
R17	Ig kappa chain V-IV region	110	3	Up regulation	
R18	Hemoglobin subunit beta	81	4	Up regulation	Cytoplasm
H19	Carbonic anhydrase 1	122	6	Up regulation	Cytoplasm
R20	LIM domain and actin-binding protein	40	1	Up regulation	cytoskeleton

Figure 2. Western blot analysis of RBC Hsp70 and Carbonic anhydrase was validated in 4 normal controls and 4 HbH-CS patients.

CONCLUSION

HbH-CS is a disorder of protein misfolding, precipitation, and aggregation of unstable β and CS globin chains. The accumulation of these globin chains may potentially subject to modulation by unfolded protein response (UPR) pathway. The UPR signaling genes and proteins in HbH-CS cultured erythroblasts will be investigated. The results will improve our understanding of how the UPR pathway regulates hemoglobin homeostasis.

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