การวิเคราะห์โปรตีนรวมของเกล็ดเลือดในสภาวะการแข็งตัวของเลือดสูงเกินปกติของผู้ป่วย เบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี

นางสาวพวงผกา จันทร์เปล่ง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์โลหิตวิทยาคลินิก ภาควิชาจุลทรรศน์ศาสตร์คลินิก
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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Protein profiling analysis of platelets in hypercoagulable state of β-thalassemia/HbE patients

Miss Puangpaka Chanpeng

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Clinical Hematology Sciences
Department of Clinical Microscopy
Faculty of Allied Health Sciences
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Thesis Title	Protein profiling analysis of platelets in hypercoagulable state of β-thalassemia/HbE patients
By	Miss Puangpaka Chanpeng
Field of Study	Clinical Hematology Sciences
Thesis Advisor	Kamonlak Leecharoenkiat, Ph.D.
Thesis Co-Advisor	Associate Professor Saovaros Svasti, Ph.D.

Accepted by the Faculty of Allied Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Allied Health Sciences
(Assistant Professor Palanee Ammaranond, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Sirikalaya Brimson, Ph.D.)

.....Thesis Advisor
(Kamonlak Leecharoenkiat, Ph.D.)

.....Thesis Co-Advisor
(Associate Professor Saovaros Svasti, Ph.D.)

.....Examiner
(Supantitra Chanprasert, Ph.D.)

.....External Examiner
(ProfessorDuncan R. Smith, Ph.D.)

.....External Examiner
(Kittiphong Paiboonsukwong, M.D., Ph.D.)

พวงผกา จันทร์เปล่ง : การวิเคราะห์โปรตีนรวมของเกล็ดเลือดในสภาวะการแข็งตัวของเลือดสูงเกินปกติของผู้ป่วย เบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี (Protein profiling analysis of platelets in hypercoagulable state of β-thalassemia/HbE patients) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดรกมลลักษณ์ ลีเจริญเกียรติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. มล.เสาวรส สวัสดิวัฒน์, 77 หน้า.

โรคเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอีเป็นโลหิตจางที่เกิดจากความบกพร่องของการสังเคราะห์สายเบต้าโกลบินซึ่งได้รับการถ่ายทอดทางพันธุกรรม ส่งผลทำให้มีการสะสมสายแอลฟาโกลบินส่วนเกินในเซลล์เม็ดเลือดแดง ปัจจุบันมีรายงานอุบัติการณ์ของสภาวะการแข็งตัวของเลือดสูงเกินปกติในผู้ป่วยโรคนี้ซึ่งถือเป็นปัจจัยเสี่ยงนำไปสู่การเกิดลิ่มเลือดอุดตันและทำให้ผู้ป่วยเสียชีวิตได้ ภาวะแทรกซ้อนนี้พบมากในผู้ป่วยตัดม้าม การศึกษาก่อนหน้านี้พบว่าการเพิ่มขึ้นของการกระตุ้นเกล็ดเลือดและปัจจัยการแข็งตัวของเลือดจะพบบ่อยในกลุ่มผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี ที่มีอาการรุนแรงปานกลาง อย่างไรก็ตามข้อมูลเชิงลึกเกี่ยวกับกลไกผิดปกติในระดับโมเลกุลของภาวะแทรกซ้อนนี้ยังไม่ชัดเจน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาโปรตีนผิดปกติที่เกี่ยวข้องกับการกระตุ้นเกล็ดเลือดและสภาวะการแข็งตัวของเลือดสูงเกินปกติในผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี อาสาสมัครเข้าร่วมโครงการวิจัยประกอบด้วยผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี ที่ไม่ถูกตัดม้าม จำนวน 15 ราย ผู้ป่วยที่ถูกตัดม้ามจำนวน 8 ราย และอาสาสมัครปกติจำนวน 20 ราย ตัวอย่างเลือดของอาสาสมัครได้ถูกนำมาตรวจวัดสภาวะการกระตุ้นของเกล็ดเลือดและระดับ prothrombin fragment 1+2 การศึกษานี้ได้เปรียบเทียบโปรตีนรวมของผู้ป่วยและคนปกติด้วยเทคนิคโปรติโอมิกส์

ผลการทดลองพบระดับการกระตุ้นของเกล็ดเลือดและระดับ prothrombin fragment 1+2 ในผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอีเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับคนปกติ (p <0.05) นอกจากนี้ยังพบว่าการกระตุ้นของเกล็ดเลือดมีความสัมพันธ์กับระดับของ prothrombin fragment 1+2 (rs = 0.6146, p < 0.0018) ผลการวิเคราะห์โปรตีนรวมพบโปรตีนแสดงออกแตกต่างกันจำนวนทั้งหมด 19 ชนิด โปรตีนที่เพิ่มขึ้นส่วนใหญ่เป็นโปรตีนโครงสร้างในเกล็ดเลือดจำนวน 10 ชนิด นอกจากนี้ยังพบโปรตีนที่เกี่ยวข้องกับการสร้างพลังงานภายในเซลล์ 1 ชนิด โปรตีนที่เกี่ยวข้องกับการกระตุ้นระบบภูมิคุ้มกันในเซลล์จำนวน 1 ชนิด โปรตีนที่เกี่ยวข้องกับการสังเคราะห์ฮีมจำนวน 2 ชนิด และเอนไซม์ต้านอนุมูลอิสระ จำนวน 2 ชนิดสูงขึ้นอย่างมีนัยสำคัญในผู้ป่วย เบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี ส่วนการแสดงออกของโปรตีนยับยั้งเอนไซม์โปรตีเอสลดลงในผู้ป่วย ระดับของโปรตีน chaperone (Hsp70), fibrinogen receptor (integrin αIIb) และ chemokine (PF4) สูงขึ้นอย่างมีนัยสำคัญในผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอีเมื่อเทียบกับคนปกติและมีความสัมพันธ์เชิงบวกกับระดับเซรั่ม ferritin (p<0.05)

การศึกษานี้ค้นพบโปรตีนที่เกี่ยวข้องการกระตุ้นเกล็ดเลือดและสภาวะการแข็งตัวของเลือดสูงเกินปกติในผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี ผลการศึกษานี้จะช่วยให้เข้าใจกลไกการเกิดพยาธิสภาพ และสามารถนำไปใช้เป็นตัวบ่งชี้ภาวะลิ่มเลือดอุดตันและพัฒนาการรักษาผู้ป่วยโรคเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอีได้ต่อไปในอนาคต

ภาควิชา จุลทรรศน์ศาสตร์คลินิก ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาศาสตร์โลหิตวิทยาคลินิก ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5776660837 : MAJOR CLINICAL HEMATOLOGY SCIENCES
KEYWORDS: PLATELET PROTEOMICS/ β-THALASSEMIA/ PLATELET ACTIVATION/ HYPERCOAGULABLE STATE/ PROTEOMIC ANALYSIS

PUANGPAKA CHANPENG: Protein profiling analysis of platelets in hypercoagulable state of β-thalassemia/HbE patients. ADVISOR: KAMONLAK LEECHAROENKIAT, Ph.D., CO-ADVISOR: ASSOC. PROF. M.L. SAOVAROS SVASTI, Ph.D., 77 pp.

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. This study aimed to identify proteins related to platelet activation and to hypercoagulable state in the β-thalassemia/HbE patients.

Fifteen non-splenectomized β-thalassemia/HbE, 8 splenectomized β-thalassemia/HbE and 20 normal controls were recruited to determine the hypercoagulation state including detection levels of platelet activation and prothrombin fragment 1+2. The proteomic analysis was performed to compare to platelet proteome between the β-thalassemia/HbE patients and normal subjects.

The levels of platelet activation and prothrombin fragment 1+2 in β- thalassemia/HbE patients were significantly increased as compared to normal controls (p<0.05). Moreover, platelet activation strongly correlated with levels of prothrombin fragment 1+2 (rs = 0.6146, p < 0.0018). Proteomic analysis revealed a total of 19 differentially expressed proteins. Most of the up-regulated proteins are 10 platelets cytoskeleton proteins. In addition, 1 immune activation related protein, 2 proteins involved in heme and globin synthesis and 2 antioxidant enzymes were also up-regulated in the β-thalassemia/HbE patients. A protease inhibitor was found to be down-regulated in β-thalassemia/HbE. Additionally, significantly elevated expression of a chaperone protein (Hsp70), a fibrinogen receptor (integrin αIIb) and a chemokine (PF4) was observed in β-thalassemia/HbE as compared to normal controls and those proteins were positively correlated with serum ferritin levels (p<0.05).

In conclusion, proteins related to platelet activation as well as the hypercoagulable state of β-thalassemia/HbE have been described. The data from this study will lead to novel insights to understand the pathophysiological conditions in the β-thalassemia/HbE patients. The novel proteins in platelet activation may serve as predictors of the thrombosis state and improve treatment modalities for β-thalassemia/HbE patients

Department:	Clinical Microscopy	Student's Signature	.....
Field of Study:	Clinical Hematology Sciences	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

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Puangpaka Chanpeng

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Chapter I

Introduction

Thalassemias are defined as a group of inherited hemolytic anemia caused by abnormalities related to β or α globin production. In β-thalassemia, β-globin synthesis is disturbed resulting in accumulation of excess α-chains in red blood cells (RBC) cytosol (1). Hemoglobin E (β -26 Glu →Lys) is the most prevalent hemoglobin variant in Southeast Asia, particularly Thailand. Hemoglobin E (HbE) interacts with β-thalassemia phenotype in β-thalassemia/HbE showing a pathophysiological severity ranging from severe transfusion-dependent thalassemia to thalassemia intermediate (2). Although the synthesis of β-globin chains in β-thalassemia is absent or reduced, the synthesis of α-globin chains continues at normal level resulting in an excess of unmatched α-globin chains that precipitate and form inclusion bodies inside the erythroid precursors (3). The unmatched α-globin and its degradation products such as heme or hemin damage membrane structures leading to accelerated apoptosis and premature destruction of the erythroid precursors in the bone marrow which is called ineffective erythropoiesis (4, 5).

A high prevalence of hypercoagulopathy and thrombosis events such as increased platelet count and platelet activation in β-thalassemia/HbE patients has been observed (6). Activation of platelets contributes to the hypercoagulopathy state such as elevated levels of tissue factor have been demonstrated in thalassemic patients. However, the mechanism of hypercoagulopathy in β-thalassemias/HbE patients had not been fully elucidated. One way to apprehend the various reasons behind the hypercoagulability status of thalassemia subjects was to study the platelet proteome. In this study, the molecular and cellular mechanisms leading to hypercoagulation in β-thalassemia/HbE investigated, with a special focus on the pathogenesis underlying the activation of platelets in hypercoagulable state. A proteomic approach was used to investigate protein expression in peripheral blood platelets isolated from normal controls and β-thalassemia/HbE patients with and without splenectomy. An understanding of the alterations in expression of proteins in platelets may improve our understanding of the biological pathogenesis of hypercoagulation observed in β-

thalassemia/HbE disease and lead to the possibility of improved treatment modalities for β-thalassemia/HbE patients as well as better predictors of thrombosis state in these patients.

Objective

To investigate platelet protein profiling related to platelet activation and the hypercoagulable state in the β-thalassemia/HbE patients.

Chapter II

Literature Review

2.1 Thalassemia

Inherited hemoglobin disorders are classified into two different groups, namely qualitative and quantitative hemoglobin production. Qualitative problems consist of a wide range of conditions that result from the production of abnormal structural globin chains for example sickle cell anemia, hemoglobin E; quantitative problems are disorders that result from reduced output of globin production which are the thalassemia syndrome (7). The thalassemias are a common inherited disease, particularly in Mediterranean and Southeast Asia including Thailand (Figure 1, 2). This disease is characterized by a deficiency or absence of the production of one or others of the globin chains of hemoglobin (1). The defect may affect one type of globin chain, or may affect some combination of more than one type in the same patient (8). The result is causes an imbalance in the production of globin chains and the production of an inadequate number of red blood cells with abnormal morphology (9). Thalassemias are classified base on defective globin chain into two main types including α and β-thalassemia (10). Moreover, the severity of clinical pathophysiology is divided into three categories. Thalassemia major (TM) such as hemoglobin Bart's hydrops fetalis, β^0/β^0 and β^0/β^+, is a transfusion dependent subtype. Thalassemia intermedia (TI) such as hemoglobin H (HbH) and HbE/β^0/ β^+, is a non-transfusion dependent thalassemia. Thalassemia minor including heterozygous of α or β thalassemia such as -α/αα, --/αα, β/β^0, β/β^+ (11).

2.1.1 α-thalassemia

The α-thalassemias are the most prevalently distributed hemoglobinopathies in Thailand (12, 13). The α-thalassemia arises from defects in α-globin chain production (14). There are two main varieties of α-thalassemias, namely deletion and non-deletion. Alterations of α-globin genes are largely caused by a deletion of one or more of these genes. A single gene deletion (-α/αα) results in α-thalassemia silent carrier status. Two gene deletions (--/αα or -α/-α) causes alpha thalassemia trait (minor) with microcytosis

and usually no anemia. The three genes deletion (--/-α) which leave only one functional α-globin gene results in a significant production of hemoglobin H (HbH) (15). Four gene deletions (--/--) result in significant production of hemoglobin Bart's (Hb Bart's) that usually results in fatal hydrops fetalis (16). In other types, non-deletional types caused by point mutation or small insertion have also reported. The most common non-deletional α-thalassemia detected in Thai population included the Hb Constant Spring (Hb CS), Hb Suan Dok and Hlb Mahidol (Hb Q) variants (17).

2.1.2 β-thalassemia

β-thalassemias are a quantitative reduction of β-globin chains that are usually structurally normal. It is caused by over 200 point mutations that nearly all affect the β globin locus and extremely heterogeneous. Almost every possible defect affecting gene expression at transcription or post-transcriptional level, including RNA processing and translation have been identified in β-thalassemia. Diversity of variably genetic leads to a variable reduction in β-globin output from a minimal deficit (mild β^{+}-thalassemia) to complete absence (β^{0}-thalassemia) causing clinically heterogeneous. The β^{0}-thalassemia has no production of β-globin chains. Individuals homozygous for this abnormal gene produce only HbA_2 and HbF with unstable α_4 tetramers that are toxic to the erythroid progenitors (5, 18). The β^{+}-thalassemia allows some of β-globin chains and patients homozygous for this gene still have trouble with the effects of an excess a globin on the erythroid progenitors in the marrow (5, 19).

2.1.3 β-thalassemia/HbE disease

Hemoglobin E (HbE) is one of the world's most common Hb variants and is mostly prevalent in and around Thailand. HbE is caused by a single base mutation of β-globin gene at codon 26, a GAG to AAG substitution, which leads to amino acid change from glutamine to lysine as a result of structurally abnormal hemoglobin, as well as an activation of a cryptic splice site. The aberrantly spliced β^{E}-globin mRNA resulted from cryptic splice site contains 16 nucleotides deletions of exon I. This leads to the alteration of reading frame and consequently, translated into the non-functional

products, thereby causing a reduction of β^{E}-globin chains synthesis. Therefore, β^{E}-globin gene could lead to β^{+}-thalassemia phenotype (20, 21).

The most common compound heterozygous state for the β-thalassemia is β-thalassemia/HbE disease which is frequently seen in parts of the Indian subcontinent and throughout Southeast Asia. In Thailand (22, 23), there have been approximately 3,000 births with the β-thalassemia/HbE since 1989 (24). One critical complication of β-thalassemia/HbE is patients with apparently similar or identical genetic defects showing different severity of presentation (22). To date, the severity classification of the β^{0}-thalassemia/HbE is mostly based upon the analysis of basic hematological parameters combined with the patient's clinical presentation. The patients with β^{0}-thalassemia/HbE are classified into mild, moderate and severe cases. In a review of 950 Thai β^{0}-thalassemia/HbE patients, 30 % were mild, 37 % were moderate and 33 % were severe cases (25).

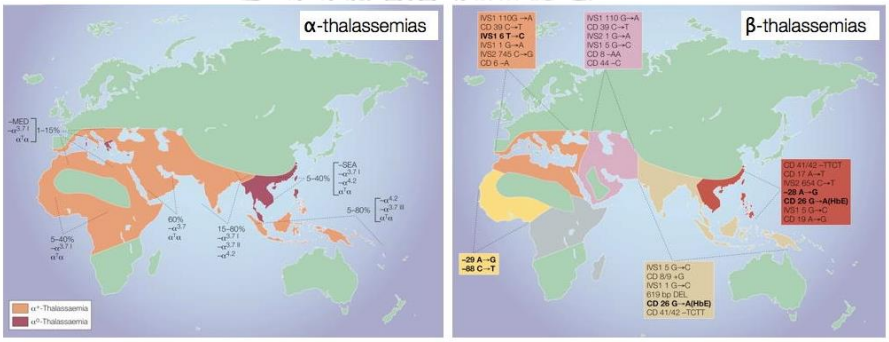

Figure 1Schematic of worldwide distribution of α and β-thalassemia patients

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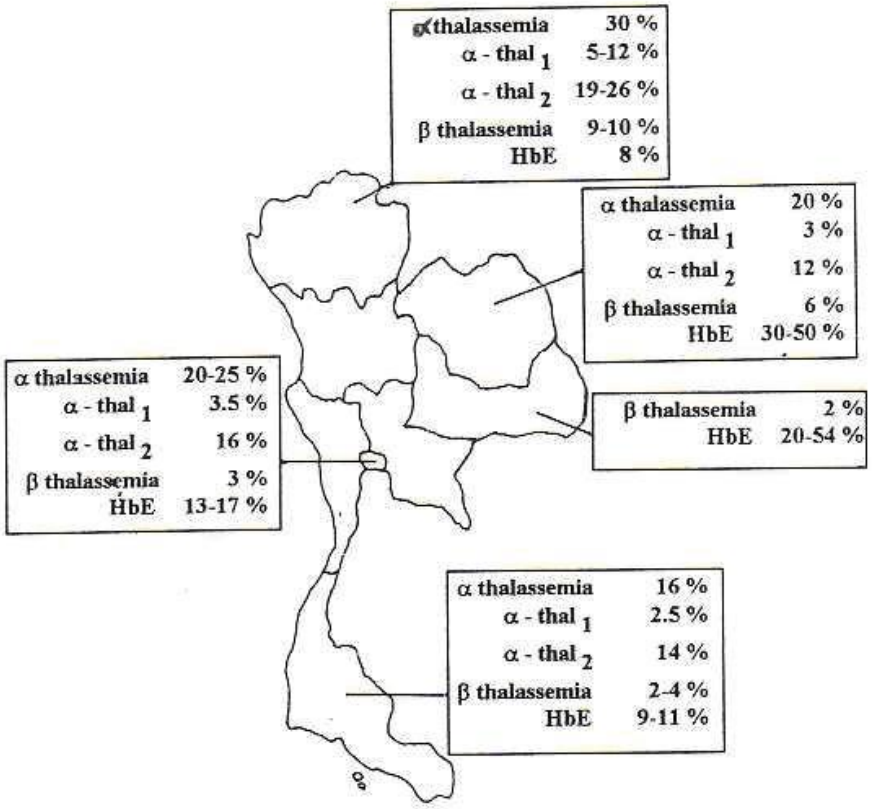

Figure 2 The prevalence of thalassemia in Thailand.

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2.1.4 Pathophysiology of β-thalassemia/HbE disease

An imbalance of α-/β-globin chain synthesis, results in an excess of unmatch α-globin chains, which are deposited in β-thalassemic erythroid precursors, mainly in the polychromatophil stage of differentiation (27). Excessive of α-globin chains can form irreversible hemichromes (28). Hemichromes tend to precipitate in RBCs and bind to various components of the membrane, such as protein band 3, protein 4.1, ankyrin, and spectrin (29-31). In addition, after precipitation, hemichromes and heme disintegrates, resulting in a production of reactive oxygen species (ROS) through Fenton reaction, the process that free iron (II) is oxidized by hydrogen peroxide resulting in presence of hydroxyl radicals, a strong ROS, which may possibly to damage different biological target molecules such as DNA, proteins and lipids (29). This causes oxidative stress in thalassemic RBCs. Moreover, a study on RBC membrane skeletal protein defect in α- and β-thalassemia demonstrated oxidation of membrane proteins on RBCs (29-31). They found evidence of oxidation of protein 4.1 in β-thalassemia (31). Protein 4.1 functions are not only form to the spectrin-actin-protein 4.1 lattice of the membrane skeleton, but also acts as a possible anchoring point of the skeleton with transmembrane proteins by interacting with glycoprotein C. RBC membranes deficient of defective in protein 4.1 are mechanically unstable. The oxidized protein 4.1 in β-thalassemia membranes could explain membrane instability (31). The thalassemic RBCs are abnormal distribution or loss of plasma membrane asymmetry by translocation of PS and PE from inner leaflet into outer leaflet and move phosphatidylcholine (PC) into inner leaflet (31). Significantly higher fractions of annexin V labeled RBCs were found in β-thalassemia major patients and β-thalassemia intermedia patients compared to healthy individuals (32). PS-exposing is an important signal for removal. All of this can be summarized, in that an imbalancing of α-/β-globin chain synthesis is a cause of ineffective erythropoiesis in β-thalassemia/HbE patients leading to anemia and undergo various complications in patients (18, 27, 33).

2.1.5 Clinical manifestations

The clinical manifestations of this syndrome are heterogeneous, ranging from a mild form of thalassemia intermedia to severe transfusion dependency. Complications of β-thalassemia/HbE in patients depend on the severity of patients (34), as described below.

2.1.5.1 Organ changes

Infants with β-thalassemia/HbE are usually asymptomatic because of their high HbF level (35). Symptoms first start at age of 6-12 months with anemia and abdominal enlargement when there is a decrease in the level of HbF production. The most common symptoms include anemia and splenomegaly. Mongoloid or thalassemia faces are observed in 82% of the patients, with about half of patients having apparent changes resulting from a high level of erythropoietin (Epo), which drives erythroid expansion and extramedullary hematopoiesis (27). Hepatosplenomegaly is detected in 90% of the patients (36). However, there is no correlation between the degree of organ enlargement and the state of the diseases. Full blown manifestations develop over time in the absence of blood transfusion. The patients in whom the symptoms initially appear during the first year of life usually have a more severe case of disease (34).

2.1.5.2 Iron overload

Tissue iron overload is the most important complication of β-thalassemia (27). It causes mortality and morbidity associated thalassemia (18). Iron overload in β-thalassemia results from multiple blood transfusions and an increase in gastrointestinal absorption of iron (21). Iron deposition occurs in many organs (mainly in the heart, liver, and endocrine glands), causing tissue damage and ultimately organ dysfunction and failure (36). Cardiac events due to iron overload are still the primary cause of death. For patients, excess gastrointestinal iron absorption persists despite massive increases in total body iron. Hepcidin is a key regulator of iron homeostasis. It is a small peptide that inhibits intestinal iron absorption and blocks iron release from macrophage and hepatocytes (5, 34). Hepcidin levels normally increase when iron

stores are elevated. However, hepcidin levels were found to be inappropriately low in patients with β-thalassemia intermedia and β-thalassemia major which involved with two hepcidin erythroid regulators; the growth differentiation factor 15 (GDF15) and the twisted gastrulation protein homolog1 (TWSG1) (37). These proteins down regulate hepcidin secretion by hepatocytes (27). They have been reports that serum from β-thalassemia patients inhibited hepcidin messenger RNA expression in the HepG2 call line (38). GDF15 expression is associated with cellular stress and apoptosis. In β-thalassemia erythroid differentiation, GDF15 has been shown to be secreted by apoptotic erythroid cells at final stages. In contrast, the highest levels of TWSG1 were detected at early stages of erythroblast differentiation, before hemoglobinization (27).

2.1.5.3 Bone marrow expansion

Erythropoiesis is markedly increased to 10-15 times normal due to high Epo production (39). In β-thalassemia major mice model, it was shown that persistent activation of JAK2, as a consequence of high levels of Epo, drives erythroid expansion and extramedullary hematopoiesis (39). Massive erythropoiesis is observed in the bone marrow and extramedullary sites including liver, spleen, lymph node and others. Erythropoietic masses in the spinal canal can cause spinal cord compression of convulsions when they appear intracranially. These masses respond to local radiation, which can reverse such symptoms. Massive erythropoiesis leads to bone fragility and distortion in bone density is decreases due to osteoporosis and osteomalacia (27, 34).

2.1.5.4 Endocrinopathies

Impaired of growth and endocrinopathies, particular hypogonadism, are common features of thalassemia (40). Since these manifestations result from chronic anemia, as well as from iron overload, they are much more commonly found in older patients of those whose chelation therapy is insufficient (18).

2.1.5.5 Cadiovascular complication

Heart disease is the major cause of death in about 20.7% of thalassemia patient (41). Iron overload and severe anemia are important causes of iron deposition that are associated with organ failure (4).

2.1.5.6 Infection

Infections are a major complication and cause of death in patients with β-thalassemia/HbE, especially in splenectomized cases. Prospective studies indicate increased susceptibility to bacterial, fungal and viral infections (42). Post-splenectomy overwhelming infections can be very severe and cause death. The causative organisms are mostly gram negative bacteria. The causes of increased susceptibility to infections are unknown, other than the well-appreciated immune defects found in post-splenectomized patients. No specific defect in humoral and cellular immune responses have been reported in thalassemia. However, the underlying mechanism may involve altered interaction between erythrocytes, cellular and humoral immune responses, complement and the reticuloendothelial system (34).

2.1.5.7 Hypoxemia

Hypoxemia is observed in a majority of splenectomized patients. The underlying mechanism is unknown but a hypothesis is that platelets, which are increased and may be more associated after splenectomy, aggregate in the circulation and in the pulmonary vasculature (43). This platelet aggregation may release a substance that causes bronchiolar constriction leading to decreased oxygenation. Consistent with this hypothesis is preliminary studies that have indicated that the administration of aspirin can ameliorate the degree of hypoxemia in the majority of cases (34).

2.1.5.8 Splenectomy

The main indications for splenectomy in thalassaemia intermedia are a significant enlargement of the spleen and a decrease in mean hemoglobin levels in the

absence of other transient factors such as infection as a minimally invasive alternative that may become the treatment of choice in β-thalassaemia patients who require concurrent operations (44). Patients with thalassaemia intermedia have been shown to have an increased predisposition to thrombosis compared with thalassaemia major patients (45). These events mainly occur in the venous system and are comprised of deep vein thrombosis (40%), portal vein thrombosis (19%), stroke (9%), pulmonary embolism (12%) and others (20%) (46). Moreover, the splenectomized patients have been shown to have a higher risk of thrombosis than non-splenectomised patients (46). The major long-term risk after splenectomy is overwhelming sepsis. In previous studies, the risk of post-splenectomy sepsis in thalassaemia major is increased more than 30-fold in comparison with the normal population (47). A higher risk is seen in splenectomized patients. The phenomenon of hypercoagulability is related to the fact that damaged red blood cells normally removed by the spleen that persist in the circulation and trigger mechanisms of thrombin generation. In post-splenectomy patients markers of thrombin generation such as thrombin AT III (TAT) complexes, prothrombin fragments (F1+2) fibrinopeptide A (FPA) and D-dimer should be assessed annually, and anti-coagulant prophylaxis prescribed where indicated (44).

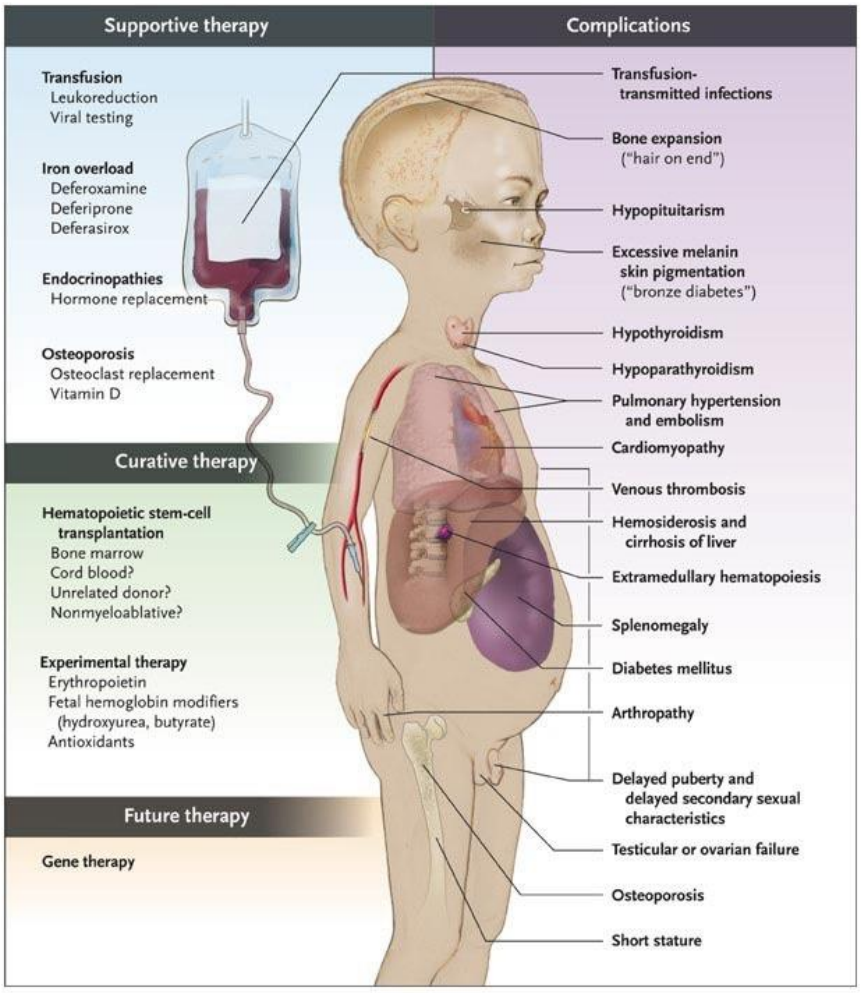

Figure 3 Complication of β-thalassemia/HbE and therapy (5).

The anemia in β-thalassemia/HbE is severe and accompanied by ineffective erythropoiesis, with bone expansion and extramedullary hematopoiesis. Transfusion therapy, which is the mainstay of treatment, allows for normal growth and development and suppresses ineffective erythropoiesis. Iron overload results from both transfusion and excess gastrointestinal iron absorption. Iron deposition in the heart, liver, and multiple endocrine glands causes in severe damage to these organs (27, 38). "Reprinted by permission from New England Journal of Medicine: Medical progress β-thalssemia, copyright 2005"

2.2 Thromboembolism and hypercoagulable state in β-thalassemia

Recently, a multicenter study was conducted in order to assess the extent of this problem in β-thalassemia patients. It is reported that 146 out of 8860 patients (1.65%) experienced thrombotic events, with a prevalence of 0.9% in β-thalassemia major and 4% in β-thalassemia intermedia (48). The highest prevalence of these events was observed in splenectomized patients (49). In splenectomized patients, plasma β-TG levels were significantly higher than in control subjects and patients who had not undergone splenectomy (50). The high incidences of thrombotic events in β - thalassemia/HbE have been reported especially in β–thalassemia intermedia patients (50). Moreover, previous evidence has shown that pulmonary thromboembolic lesions were found in 24 out of 58 (41%) patients with β-thalassemia/HbE, of which 21 out of 39 (54%) were splenectomized patients and 3 out of 19 (16%) were non-splenectomized patients (50). The β-thalassemia/HbE is associated with a deficiency of β-globin chain synthesis, leading to an accumulation of the remaining excess globin chains. This process is an associated with a loss of the normal asymmetrical distribution of the RBC membrane phospholipids and translocation of phosphatidylserine (PS) to the external membrane leaflet (flip-flop). The membrane damage is related to lipid peroxidation mediated by free iron and increased amounts of membrane-bound hemichromes and immunoglobulins and modifications in the membrane band 3 proteins and spectrin (51). The membrane changes may partly explain the enhanced aggregation of PS-exposing RBCs, their increased adherence to ECs, and their capacity to enhance thrombin generation via the assembly of the prothrombinase complex. The enhanced thrombin generation leads to activation of platelets, monocytes, granulocytes, and ECs and expression of tissue factor, which further enhances the thrombotic process (51). The low levels of the coagulation inhibitors, protein C and protein S, further facilitate the result in the hypercoagulable state. The combinations of these abnormalities lead to clinical thrombosis (51). These factors include abnormal RBC surface, platelet activation, peripheral blood elements, splenectomy, nitric oxide, thrombophilia and other factors (Figure 4) (52). Especially, for platelet activation, there is a strong piece of evidence that has shown increased platelet aggregation, an increased proportion of platelets expressing CD62P (P-selectin) and CD63, and a shortened platelet survival

due to enhanced platelet consumption. This is especially true in splenectomized patients (53). In addition, the hemostatic abnormality, including lower protein C, protein S, and the significantly elevated level of thrombin anti-thrombin (TAT), F1+2 and D-Dimer complexes. In addition, chronic platelet activation showed the elevated plasma levels of platelet factor 4 (PF4) and β-thromboglobulin (β-TG) in splenectomzied β-thalassemia/HbE (51). All of this causes chronic platelet activation which leads to thromboembolism phenomena, both venous and arterial. The proposed model of mechanism involving the hypercoagulable state in β –thalassemia was established (Figure 5).

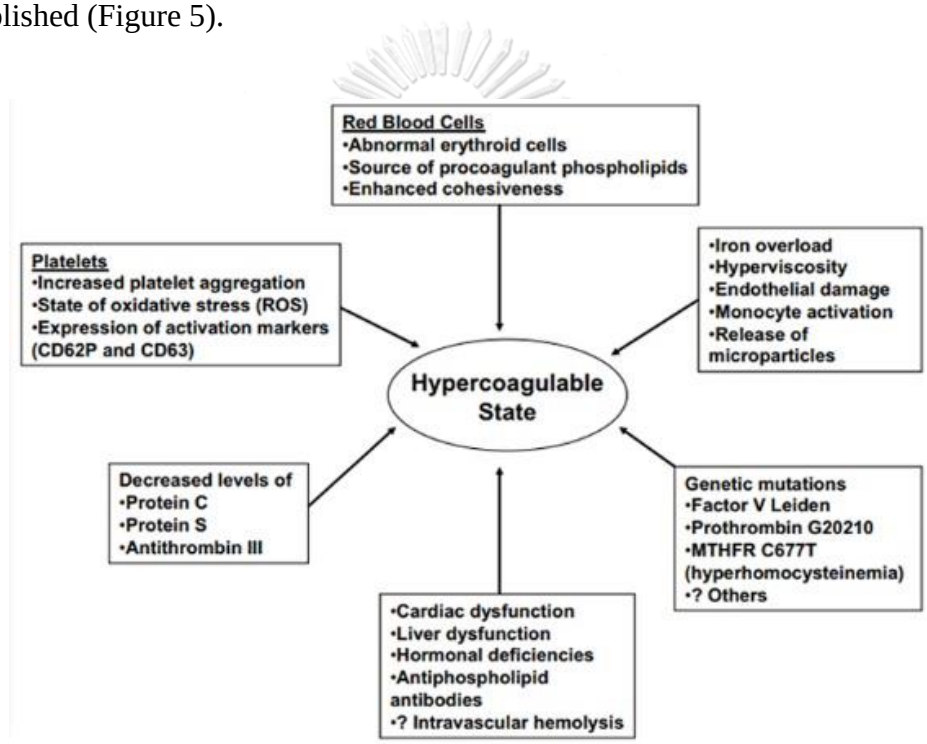

Figure 4 Factors contributing to the hypercoagulable state in thalassemia (52).

There are a diverse range of factors contributing to the hypercoagulable state in patients with β-thalassemia. In most cases, a combination of these abnormalities leads to clinical thrombosis. **“**Reprinted by permission from Blood reviews, Vol 22, Issue 5, Pages 283-292, Ali T. Zaher K. Otrock, Imad Uthman, Maria D. Cappellini, Thaassemia and hypercoagulability, copyright 2008”

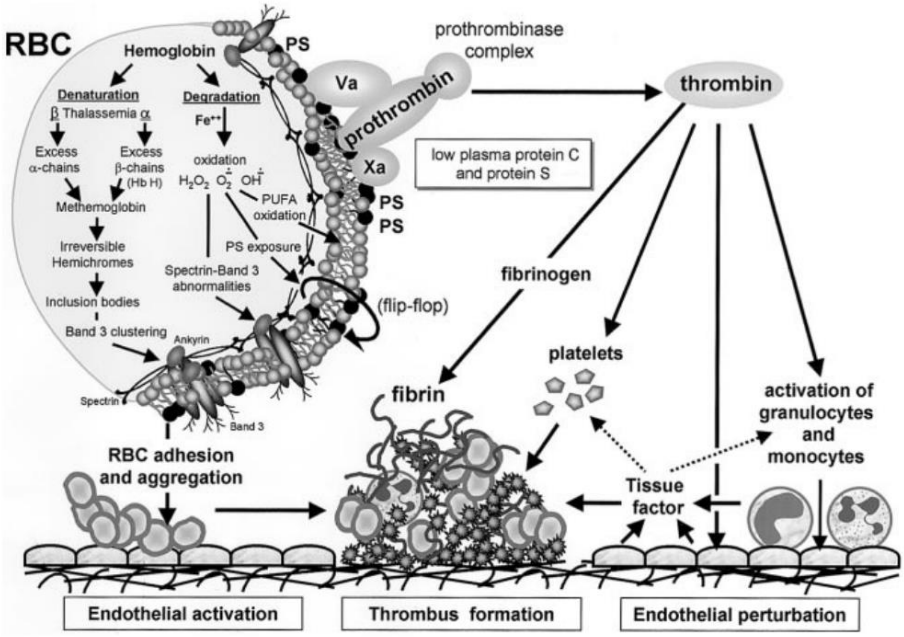

Figure 5 The hypercoagulable state in β-thalassemia/HbE (51).

Thalassemia is associated with partial or complete deficiency of α- or β-globin chain synthesis, which leads to denaturation and degradation of the remaining globin chains. This process is associated with loss of the normal asymmetrical distribution of the RBC membrane phospholipids and translocation of PS to the external membrane leaflet (flip-flop). The membrane damage is related to lipid peroxidation mediated by free iron and increased amounts of membrane-bound hemichromes and immunoglobulins and modifications in the membrane band 3 proteins and spectrin. The membrane changes may partly explain the enhanced aggregation of PS-exposing RBCs, their increased adherence to ECs, and their capacity to enhance thrombin generation via the assembly of the prothrombinase complex. The enhanced thrombin generation leads to activation of platelets, monocytes, granulocytes, and ECs and expression of tissue factor, which further enhances the thrombotic process (51). “Reprinted by permission form Blood Journals: The hypercoagulable state in thalassemia, copyright 2002”

2.3 Platelets hemostasis

Platelets play an important role in hemostasis. In 1865, Max Schultze became the first person to discover and describe these spherules which are much smaller than red blood cells that occasionally clump and may participate in collections of fibrous material (54). At a later time, in 1882, Giulio Bizzozero observed the “spherules” microscopically in the circulating blood of living animals and in blood removed from blood vessels. He showed the first component of blood to adhere to damaged blood vessel walls in vivo, and that in vitro, they were the first blood components to adhere to threads that subsequently became covered with fibrin. These initial discoveries are at the foundation of our current understanding of hemostasis (54).

Hemostasis is a critical physiological process used to stop bleeding. Platelet accumulation at the site of an injury is considered as the first wave of hemostasis and the second wave of hemostasis is mediated by the blood coagulation pathway. Platelets play a central role of sequential events during platelet accumulation (i.e. platelet adhesion, activation, and aggregation) and are also actively involved in cell-based thrombin generation, which markedly amplifies the blood coagulation cascade. Thus, platelets contribute to both the first and the second waves of hemostasis (55-57) (Figure 6).

2.3.1. Platelet adhesion

Platelet adhered to the injured vessel wall can occur at both low and high shear conditions. Following vascular injury, subendothelial matrix proteins such as collagens are exposed to the blood components (58). Plasma von Willebrand Factor (VWF), originated from endothelial cells, megakaryocytes, and platelets, can then anchor onto the collagen. The VWF receptor on platelets (glycoprotein GPIba) subsequently initiate platelet tethering to the site of injury (59). This binding process is essential for platelet adhesion at high shear although the GPIba-VWF interaction contribute to platelet adhesion at low shear. Following platelet tethering, GPVI and integrin a2b1 interact with collagen and deliver activation signals to platelets (60). Stable adhesion is subsequently mediated by the binding of several integrins to their ligands on the vessel wall such as integrin aIIb3 to fibrinogen/fibrin. At low shear the interactions between

platelet integrins and their ligands may directly initiate platelet adhesion. In the last decade there have been significant advances in in-vivo models of platelet adhesion and thrombus formation using intravital microscopy (61).

2.3.2 Platelet activation and granule secretion

The primary interactions between platelet surface receptors (GPIba, integrins) and their ligands (VWF, collagen, fibrinogen/fibrin, fibronectin) lead to platelet activation (57, 62). In addition, following vascular injury, the coagulation system is activated which generates the most potent platelet activation factor, thrombin. Through cleavage of protease-activated receptors (PARs) and binding to GPIba, thrombin activates platelets (56). Platelet activation exposes PS on the membrane surface that drives the cell-based thrombin generation and facilitates further platelet activation (63). Activation signals induced by thrombin, collagen, or ligands of adhesion receptors with the addition of shear stress, can lead to platelet granule release. Platelet adhesion molecules, P-selectin, integrins, VWF, fibrinogen, fibronectin, vitronectin, multimerin, platelet factor 4 (PF4), and approximately 300 other proteins are contained within the α-granules (64). Dense granules release adenosine di-phosphate (ADP). The release of Ca2+ from the endoplasmic reticulum and the dense granules via the Ca2+ sensor, stromal interaction molecule (STIM), and the Ca2+ channel, is also a significant contributor to platelet activation (60). There are many positive feedback loops during platelet activation/granule release. These secretion events act as secondary messengers and, in combination with the generation of thromboxane (Tx) A2 and reactive oxygen species, amplify the activation process and integrin inside-out signaling and turn recruits platelets for aggregation. For the platelet activation, on the intracellular level, the stimulation of platelets causes phosphorylation of specific proteins via an activation of specific kinases such as myosin light chain kinase, phospholipid-dependent protein kinase 1, reorganization of cytoskeletons, an increase in actin polymerization accompanied by the association of specific proteins with newly formed actin filaments, and increased contractile activity. The phosphorylation of specific platelet proteins after activation appears to be directly related to both changes in cytoskeleton structure and the secretion of granule contents (60).

2.3.3 Platelet aggregation: fibrinogen-dependent and independent aggregation

After, platelet activation, integrin aIIbb3 binds fibrinogen and other ligands leading to platelet aggregation (60). The integrin aIIbb3 have ability to deliver outside-in signals to enhance platelet activation, cytoskeleton rearrangement and granule secretion (65). These signals facilitate hemostatic plug and thrombus formation. Moreover, fibrinogen was considered as a requirement for platelet aggregation. Through interaction with aIIbb3 via its C chain C-terminus, fibrinogen bridges activated platelets (57, 66).

2.3.4 Platelet-mediated cell-based thrombin generation and blood coagulation

The central roles in the platelet adhesion, activation, and aggregation (the first wave of hemostasis), platelets also contribute to coagulation pathway (the second wave of hemostasis) (60). The blood coagulation cascade were activated by either the extrinsic (tissue factor) or the intrinsic (contact activation) pathways in thrombosis. Thrombin, a vital product of the coagulation cascade, converts fibrinogen to fibrin, the end product of the coagulation cascade (60, 63). Besides two classical coagulation pathways, the exposure of PS on platelets, following platelet activation, markedly potentiates thrombin generation by inducing a negatively charged surface that harbors the coagulation factors (51). Recently, GPVI was also identified as a novel fibrin receptor involved in potentiating thrombin generation. Thrombin initiates robust downstream signaling, through PAR1, PAR4 and GPIba, leading to platelet activation and further PS exposure, a positive feedback loop for thrombin generation and blood coagulation. There are many interactions between the first wave (platelet accumulation) and the second wave (blood coagulation) of hemostasis, which synergistically contribute to the arrest of bleeding (60).

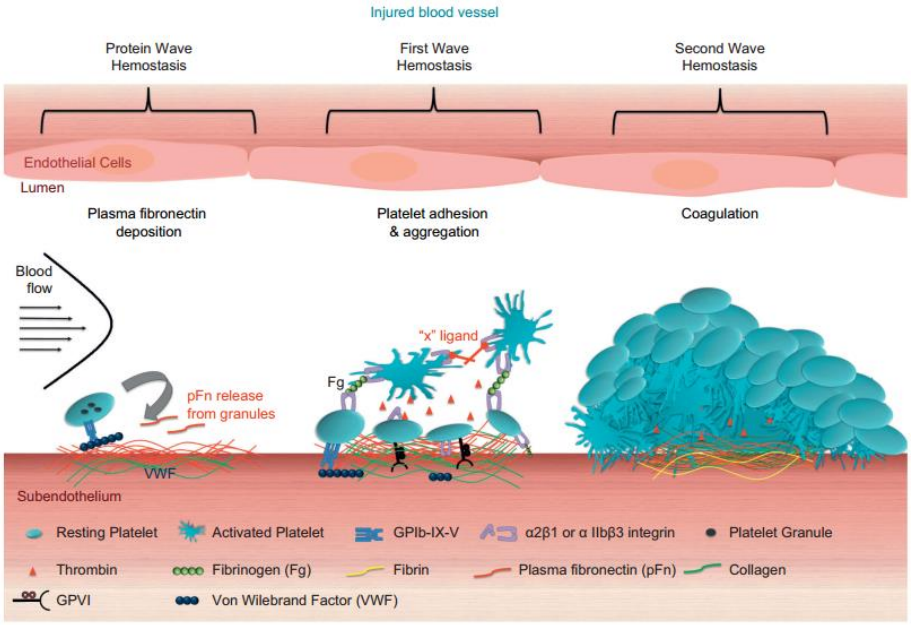

Figure 6 Schematic of platelet formation in hemostasis (60).

Platelets release their internalized plasma fibronectin from intracellular granules. Platelet receptors bind physiological ligands, such as VWF and collagen, activating integrin aIIbb3, which results in fibrinogen binding and subsequent platelet aggregation. Thrombin is generated on the negatively charged platelet surface and further activates platelets and contributes to the coagulation cascade. In a growing hemostatic plug/thrombus, the fibrin and fibronectin matrix is usually formed at the interface between the injured vessel wall and the platelet plug. “Reprinted by permission from The Journal of Biomedical Research: Platelets in hemostasis and thrombosis: Novel mechanisms of fibrinogen-independent platelet aggregation and fibronectin-mediated protein wave of hemostasis, copyright 2015”

2.3.5 Platelet activation in β-thalassemia/HbE patients.

There has been medical literature about platelet functions in hypercoagulable state of β-thalassemia/HbE which has shown strong evidence suggesting that patients with β-thalassemia/HbE have activated platelets which has been attributed to several factors such as PS-RBCs, hemolysis, microparticles, splenectomy, transfusion and iron overload, causes of oxidative stress leading to platelet activation which further enhances the thrombotic process (67, 68). In 1992, Bunyaratvej A et al reported that platelets from patients with β-thalassemia/HbE were examined in comparison to those from normal subjects by scanning electron microscopy and the results showed that the splenectomized patients had lower platelet pseudopod reversibility than non-splenectomized patients (69). This also suggested that impaired reversibility of platelet pseudopods may be associated with the high tendency of pulmonary thrombus in β-thalassemia/HbE patients (69) (Figure 7). It is widely accepted that patients with thalassemia have chronically activated platelets and enhanced platelet aggregation, as confirmed by the increased expression of CD62P (P-selectin) and CD63, markers of in vivo platelet activation. Splenectomized thalassemia patients have high platelet counts but platelets have a shorter life span due to enhanced consumption (70). The examination of circulation platelet aggregation showed increasing levels of circulating platelet aggregates in 71% of splenectomized and 35% of non-splenectomized patients with β-thalassemia disease (71). Further evidence for the existence of platelet activation in thalassemia was provided by the measurement of urinary metabolites of thromboxane A_2 (TXA_2) and prostacyclin 2 (PGI_2). A study of splenectomized β-thalassemia patients who received occasional blood transfusions and healthy individuals found a significant 4-to 10-fold increase in the urinary excretion of 2, 3-dinor-TXB2, 11-dehydro-TXB2, and 2,3-dinor-6-keto-prostaglandin F1β in β-thalassemia patients compared to healthy controls. The results of this study are consistent with enhanced production of TXA2 due to chronic endogenous platelet activation and reflect the increased concentrations of urinary thromboxane metabolites found in other diseases associated within vivo platelet activation (51). A recent report showed the cellular origin of microparticles that mostly activated-platelet origin with adhesion (CD41a/CD62P/CD36). The platelet microparticles were analyzed by flow cytometry and showed splenectomized β-

thalassaemia/HbE patients had significantly higher levels of platelet microparticles than non-splenectomized β-thalassaemia/HbE patients which reflected the existence of chronic platelet activation and were commonly seen in splenectomized β-thalassaemia/HbE patients (72, 73).

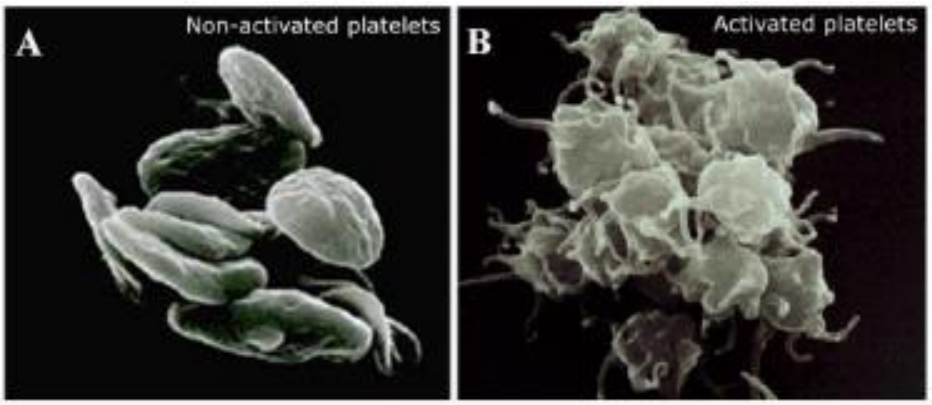

Figure 7 Resting platelet and activated platelet by scanning electron microscopy (74).

Resting platelet (A) are of a smooth disc shape**,** Activated platelet (B) have an irregular shape with many protruding pseudopodia. “Reprinted by permission from European Journal of Nanomedicine: Blood cell changes in complement activation-related pseudoallergy, copyright 2015”

2.3.6 Platelet activation and oxidative stress in β-thalassemia/HbE patients

The hallmarks of β-thalassemia/HbE are ineffective erythropoiesis and peripheral hemolysis from excess α-globin chain lead to several of subsequent pathophysiologies. In ineffective erythropoiesis, iron overload and oxidative may be promoted damage to cells and tissues that lead to a number of serious clinical mortalities and morbidities (75)**.** Iron free radicals from iron overload is suspected to activate the platelet oxidative stress. There are three main mechanisms by which iron overload develops in thalassemia; increase iron free radical due to ineffective erythropoiesis, RBC hemolysis, blood transfusions and increase dietary iron absorption to induced erythropoiesis (76, 77). The ROS affect platelet function and promote platelet activation by directly; superoxide and indirectly; nitric oxides inhibition

contributed to platelet aggregation and thrombus formation (78, 79). As seen in the previous observations, β-thalassemia/HbE patients have a higher level of serum ferritin that indicates to accumulation of iron free radicals and those free radicals could stimulate oxidative change in the platelets. Moreover, previous studies have shown that the platelet activation in β-thalassemia/HbE patients correlated with oxidative stress proteins such as heat shock protein, peroxiredoxin and integrin protein. This is especially the case, in splenectomized β-thalassemia/HbE patients (80, 81)**.**

2.3.7 Platelet proteome

Platelets are small cell fragments, produced by megakaryocytes, in the bone marrow. They play an important role in hemostasis and diverse thrombotic disorders. They are implicated in several pathophysiological pathways, such as inflammation or wound repair. In blood circulation, platelets are activated by several pathways including sub-endothelial matrix and thrombin, triggering the formation of the platelet plug (82). Studying the platelet proteome is a powerful approach to better understanding their biology and function. Proteomic experiments begin with a protein mixture which is digested to a peptide mixture, either in a gel or in solution. Two-dimensional electrophoresis (2-DE) has been available since the 1970s, and was used in the 1990s to construct early maps of platelet proteins (83). Newer gel-based methods include differential in-gel electrophoresis (DIGE), which allows for quantitation and comparison of samples from different proteomic states. In DIGE, samples are differentially labeled with fluorescent dyes and simultaneously analyzed in a single gel. Alternatively, protein spots may be excised from a gel, digested, and analyzed by mass spectrometry (MS). Gel-free methods, which use in-solution proteolytic digestion, can detect proteins not well represented by 2-DE, such as transmembrane and basic proteins. Multidimensional protein identification technology (MudPIT) employs ion-exchange and reverse-phase liquid chromatography (LC) for peptide separation. Mass spectrometry (MS) has the capacity to identify proteins in a high throughput manner, using bioinformatics approaches linked to protein sequence databases. The two main mass-spectrometric options are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Both MALDI and a related method, surface-enhanced laser desorption/ionization (SELDI), employ a matrix to ionize proteins; SELDI uses

protein chips with chromatographic surfaces. LC-MS/MS is the method now chosen by most proteomics researchers. Isotope affinity tagging techniques help to increase the sensitivity of detection of smaller peptide fragments and provide quantitative information about protein concentrations. Isotope coded affinity tags (ICAT) selectively label cysteine residues of peptide fragments following the tryptic digestion of the protein sample; this technique increases the depth of protein coverage but is limited by selective labeling. Isotope tags for relative and absolute quantitation (iTRAQ) label every tryptic fragment (84). However, particular attention must be paid to different experimental parameters, such as platelet quality and purity. Several technologies are involved during the platelet proteome processing, yielding information on protein identification, characterization, localization, and quantification. Recent technical improvements in proteomics combined with inter-disciplinary strategies, such as metabolomics, transcriptomics, and bioinformatics, will help to understand platelets biological mechanisms (Figure 8) (85, 86). The proteomic data have been published for whole proteome, membrane proteome, microparticles proteome, phosphoproteome. secretome, granule proteome, glycoproteome (Figure 9). Identification of proteins can be analyzed by pathway analysis to detect the molecular pathways mainly altered. This platelet proteomic approach can be applied to study platelet-related diseases, discover novel drug targets or biomarkers.

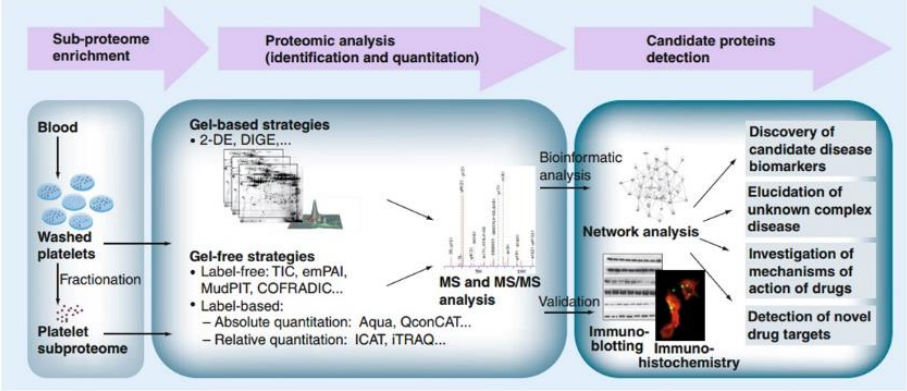

Figure 8 Summary of various proteomics workflows applied to platelet study (87).

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Chapter III

Material and Method

3.1 Material

3.1.1 Instruments and laboratory supplies

- Automated cell counter (ADVIA210, Bayer, Tarrytown, NY, USA)
- FACSCalibur flow cytometer (BD Bioscience, San Jose, CA)
- Centrifuge (Thermo Fisher Scientific, USA)
- Micro High speed refrigerated centrifuge (VISION SCIENTIFIC CO.,LTD, Korea)
- Mini centrifuge (Labnet, Toronto, Ontario, Canada)
- Hemacytometer (Blood counting chamber, BOECO, Hamburg, Germany)
- Light microscope (Olympus Optical Co., Ltd, Tokyo, Japan)
- Sonicator (Mettler Electronics Corp., Anaheim, CA, USA)
- Ettan IPGphor 3 (GE healthcare Life sciences, UK)
- miniVE vertical electrophoresis system (GE healthcare Life sciences, UK)
- image scan (GE healthcare Life sciences, UK)
- 15 mL tube centrifuge tube (Corning Incorporated, Grand Island, NY, USA)
- 50 mL tube centrifuge tube (Corning Incorporated, Grand Island, NY, USA)
- 1.5 mL microcentrifuge tube (Nest Biotechnology Co., Ltd. New District, Wuxi, China)
- 0.2 mL mocrocentrifuge tube (Nest Biotechnology Co., Ltd. New District, Wuxi, China)

3.1.2 Chemicals and reagents for platelet isolation and ELISA kits

- Citrate dextrose acid, ACD (Sigma-Aldrich Co. LLC., USA)
- Prostaglandin E1 (CAYMAN CHEMICAL, East Ellsworth Road, Ann Arbor, Michigan, USA)
- Phosphate buffer saline pH 7.4 (Gibco by life technologies, USA)
- Tyrode’s buffer (Sigma-Aldrich Co. LLC., USA)

- 1 M HEPES (Santa Cruz Biotechnology, Inc., USA)
- Human Prothrombin Fragment 1+2 Elisa kit (MyBioSource, Inc., San Diego, CA, USA)

3.1.3 Chemicals and reagents for 2D and western blot analysis

- Acrylamide (Bio Basic, Inc, Markham, Ontario, Canada)
- Bis- Acrylamide (Sigma Chemical Company, St. Louis, MO, USA)
- Ammonium persulfate (APS) (Bio Basic, Inc, Markham, Ontario, Canada)
- Tetramethylethylenediamine (TEMED) (Bio Basic, Inc, Markham, Ontario,Canada)
- Sodium dodecyl sulfate (SDS) (Bio Basic, Inc, Markham, Ontario, Canada)
- Bradford reagent (BIO-RAD, Hercules, CA, USA)
- Bromophenol blue (Bio Basic, Inc, Markham, Ontario, Canada)
- Bovine serum albumin, BSA fraction V (Sigma-Aldrich, USA)
- 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) (Bio Basic, Inc, Markham, Ontario, Canada)
- Dithiothreitol (DTT) (Bio Basic, Inc, Markham, Ontario, Canada)
- Iodoacetamide (IAA) (GE healthcare Life sciences, UK)
- Human protease inhibitor cocktail (Bio Basic, Inc, Markham, Ontario, Canada)
- Glycine (Bio Basic, Inc, Markham, Ontario, Canada)
- Tris base (Bio Basic, Inc, Markham, Ontario, Canada)
- Thiourea (Research Organics Inc., Cleveland, OH, USA)
- Urea (Research Organics Inc., Cleveland, OH, USA)
- Glycerol (Ameresco, solon, OH, USA)
- Phosphoric acid (Merck KgaA, Darmstadt, Germany)
- Methanol (Honeywell Burdick & Jackson, Cheoyong-ro, Nam-gu Ulsan, Korea)
- Coomassie blue G250 (Bio Basic, Inc, Markham, Ontario, Canada)
- Ponceau S dye (Robbin scientific, Sunnyvale, CA)
- Tween 20 (Ameresco, solon, OH, USA)
- Non-fat dry milk powder (Fonterra Brands, Thailand)

- Nitrocellulose membrane 0.2 µM pore (Millipore Corpoation, Bedford, MA, USA)
- BLUeye Prestained Protein Ladder (Bio-Helix Co., Ltd. (GeneDireX), Taiwan)
- Immobiline Drystrip pH 3-10 NL, 7 cm (GE healthcare Life sciences, UK)
- IPG buffer (GE healthcare Life sciences, UK)
- Plusone Drystrip Cover Fluid (GE healthcare Life sciences, UK)

3.1.4 Antibodies for flow cytometry

- Fluorescien isothiocyanate (FITC)- conjugated mouse anti-human platelet membrane glycoproteins (CD41a; BD biosecience, Pharmagen, San Diago, CA, USA)
- R-phycoerythrin (PE)- conjugated mouse anti-human activated human platelet (CD62P; BD biosecience, Pharmagen, San Diago, CA, USA)
- Allophycocyanin (APC)- conjugated mouse anti-human glycopholin A monoclonal antibody (CD235a; BD biosecience, Pharmagen, San Diago, CA, USA

3.1.5 Antibodies for western blot analysis

- Rabbit anti-PF4 polyclonal antibody (ab9561; Abcam, Cambridge, UK)
- Rabbit anti-integrin αIIb monoclonal antibody (D8V7H; Cell signaling Technology, Danvers, MA, USA)
- Rabbit anti-Hsp70 polyclonal antibody (sc-1060-R; Santa Cruz Biotechnology Inc, Texas, USA)
- Mouse anti-GAPDH monoclonal antibody (sc-32233; Santa Cruz Biotechnology Inc, Texas, USA)
- HRP-linked goat anti-rabbit IgG polyclonal Antibody (#7074s; Cell signaling Technology, Danvers, MA, USA)
- HRP-linked rabbit anti-mouse IgG polyclonal antibody (Pierce, Rockford, IL, USA)

3.2 Methods

3.2.1 Blood sample collection of β-thalassemia/HbE and normal controls

The study was approved by the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University. All β-thalassemia/HbE peripheral blood samples were screened into a non-splenectomized patients group and a splenectomized patients group using medical records from Nakornpathom Hospital, Thailand. All subjects above 18 years old without blood transfusion for at least a month were enrolled in this study. Patients who had hemoglobin levels less than 6 g/dL, those treated with anti-platelet drugs, and pregnant women were excluded. Complete blood counts and RBC indices of both β-thalassemia/HbE patients and normal controls were determined by an automated cell counter (ADVA210, Bayer, Tarrytown, NY, USA), providing the parameters of the red blood cell count, hemoglobin level, hematocrit, MCV, RDW, and platelet count. Hemoglobin typing was analyzed by automated HPLC system (VARIANT^{TM}, Biorad, Hercules. CA, USA) and serum ferritin levels were determined using a Ferritin Elisa kit. The numbers of samples used in each experiment are shown in Table 1.

Approximately 20 mL of peripheral blood of 20 normal controls and 23 β-thalassemia/HbE were collected and divided for each experiment as shown in Table 2. Three milliliters from EDTA blood tubes were centrifuged and the plasma was collected to measure the coagulation factor by ELISA test. The complete blood counts (CBC) and hemoglobin typing were determined from the second tube. 3 mL of a clotted blood tube were centrifuged at 3500 rpm for 15 min at 25 °C to separate the plasma in order to measure the serum ferritin. Moreover, 10 mL of citrate dextrose acid (ACD) was collected by using a double syringe technique and the platelets were isolated by the centrifugation method.

Table 1 Summarizing number of normal control and β-thalassemia/HbE samples used in each experiment

Experiment	Normal controls	Non-splenectomized patients	Splenectomized patients	Total samples
• Hematological parameters of blood samples	20	15	8	43
• Platelet function by Flow Cytometry	20	15	8	43
• Prothrombin fragment 1+2 test by ELISA	20	15	8	43
• Protein profiling between normal controls and β-thalassemia/HbE patients	4	4	-	8
• Western blot analysis	8	8	5	21

Table 2 Summarizing of anticoagulant blood tube used for each experiment

Anticoagulant tubes	Volume of blood sample (mL/tube)	Type of blood component	Test
 EDTA	3	Whole blood	• Complete blood cell count • Hemoglobin typing
 EDTA	3	Plasma	• Prothrombin Fragment 1+2 by ELISA test
 Clotted blood	3	Serum	• Serum ferritin
 ACD	10	Whole blood	• Platelet isolation

3.2.2 Platelet isolation by centrifugation method

Ten milliliters of a citrate dextrose blood was centrifuged twice at 200xg for 10 min at 25 °C to collect platelet rich plasma (PRP) as shown in Figure 9. The PRP was centrifuged at 1500xg for 15 min at 25 °C to spin down the platelet pellet. The platelet pellet was washed three times and resuspended with 1 mL of 1XPBS pH 7.4. One hundred microliters of isolated platelet suspension were subjected to check the purity by flow cytometry and 900 µL of the remaining platelet suspension were centrifuged at 13,000 rpm for 10 min at 4 °C to pellet the isolated platelets and kept for further proteomic analysis.

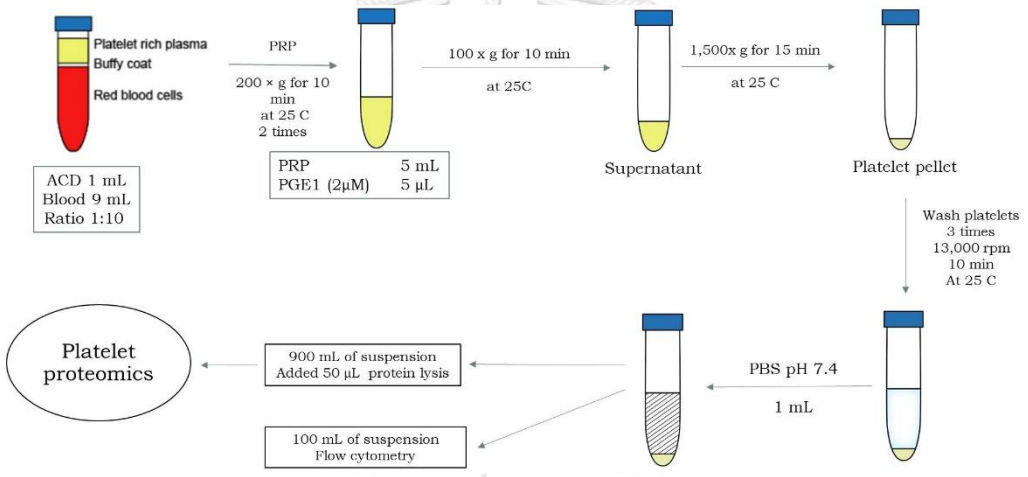


Figure 9 Schematic diagram of platelet isolation method.

3.2.3 Detection the purity of isolated platelets by flow cytometry

The purity of isolated platelets were measured by determine the levels of RBC contamination. The isolated platelet fraction was stained by adding 1 µL of anti-human GPA antibody and analyzed percentage of GPA-positive by flow cytometry. As shown in figure 10, the platelet population in R2 region was gated to analyze the percentage of contaminated RBC cells which was shown in R6 region.

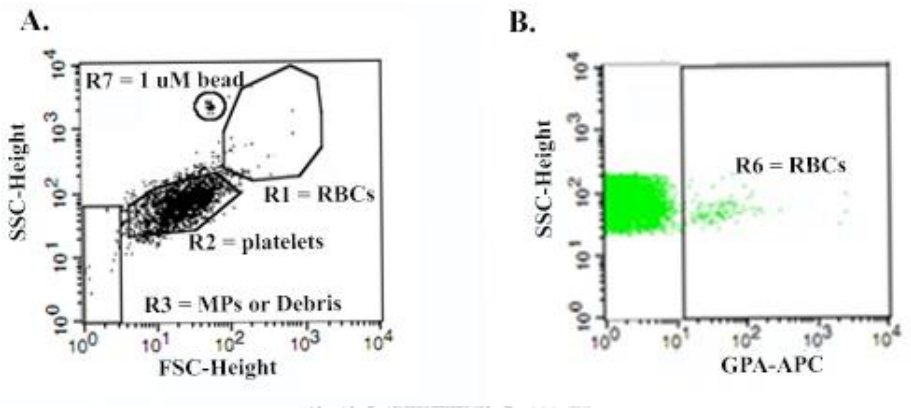


Figure 10 Measurement of contaminated RBC in platelet fraction.

The flow cytometry dot plot of isolated platelets (A) was shown in 4 populations containing RBCs population (R1), platelet population (R2), other cells as MPs and debris cells (R3) and 1 µm of Tru^{TM} count bead population calculated into absolute platelet count (R7). Moreover, the RBCs marker expression was gated from R2 into R6 (B).

3.2.4 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) and incubated for 10 min at 25 °C. After incubation the samples were centrifuged 100xg for 10 min at 25 °C to deplete the contaminated red blood cell. 10 µL of whole blood or isolated platelet were diluted with 90 µL of 1xPBS pH7.4 (dilution 1:10). 5 µL of diluted samples were transferred into FACs tubes. 1 µL of anti-human CD41-FITC, 1 µL anti-human CD62P-PE and 1 µL anti-human GPA-APC were added into the reaction tube and incubated at room temperature for 15 min in the dark. After incubated, 150 µL of PBS pH 7.4 was added in the isolated platelet FACs tube or the whole blood FACs tube and analyzed by FACs Calibur (BD Bioscience, USA). Analysis was performed by Cellquest Pro Software. The percentages of RBC and platelet population were defined by size (FSC-H), granularity (SSC-H), and flurochrome conjugated monoclonal antibody (FL1-4). 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.

3.2.5 Measurement of prothrombin fragment 1+2 levels by sandwich ELISA test

The processes of prothrombin fragment 1+2 measuring method are shown in Figure 11. Three microliters of clotted blood was centrifuged 1000xg for 15 min at 4 °C to isolate serum sample. The standard of prothrombin fragment 1+2 was diluted to 8 standard points as shown in Figure 12. An antibody specific for prothrombin fragment 1+2 was pre-coated on to a microplate and 100 µL of standards and samples were pipetted into the wells and incubated at 37 °C for 2 hrs. The present prothrombin fragment 1+2 was bound by the immobilized antibody. After removing the unbound substances, 100 µL of biotin-conjugated antibody specific for prothrombin fragment 1+2 was added to each sample well and incubated at 37 °C for 1 hour. After washing using wash buffer by Model 1575 Immunowash Microplate Washer (Bio-Rad,

California, USA), 100 µL of avidin-conjugated Horseradish Peroxidase (HRP) was added to the wells. After washing process, 90 µL of substrate solution was added to the wells and incubated at 37 °C for 25 min. 50 µL of stop solution was added to stop the color development of reaction. The color intensity of the sample reaction was measured at optical density of 450 nm by Infinite Pro M200 ELISA reader (TECAN, Switzerland) within 5 minutes. All the standards and samples measuring were done in duplicate and calculated from Curve Expert software 1.4.

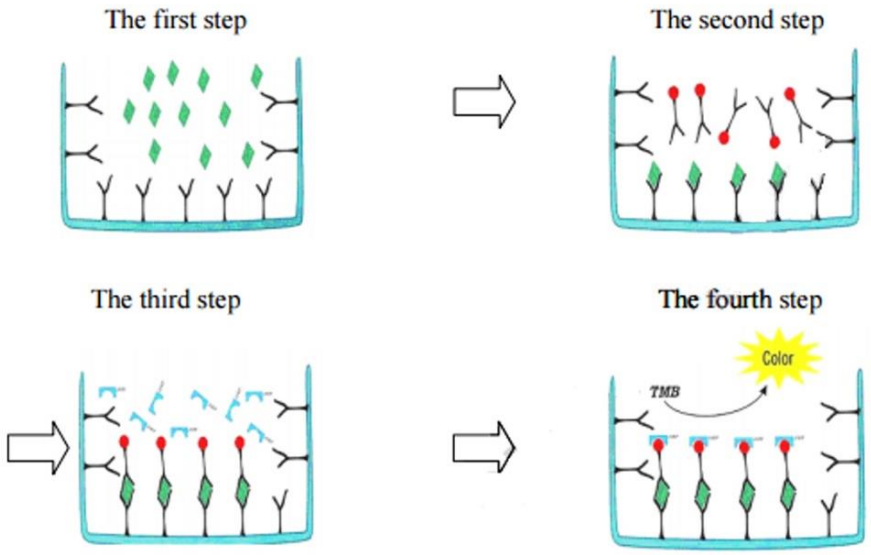
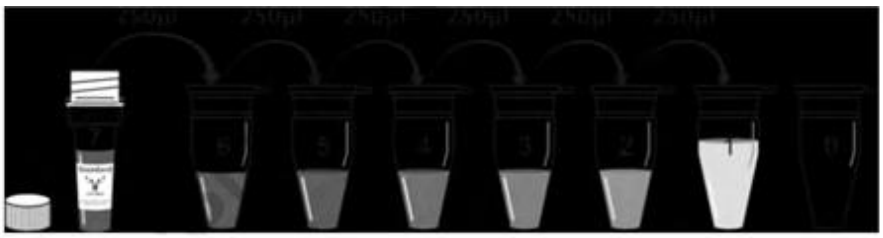


Figure 11 Schematic diagram of prothrombin fragment1+2 detected by human F1+2 ELISA kits.

Prothrombin Fragment 1+2 (MyBioSource, Inc., San Diego, CA, USA) was determined by 4 steps of sandwich enzyme immunoassay technique (88).



Tube	S7	S6	S5	S4	S3	S2	S1	S0
nmol/l	2	1	0.5	0.25	0.125	0.06	0.03	0

Figure 12 Standard dilution of prothrombin fragment 1+2.

The dilution of standard samples were prepared by 2-fold dilution for measuring the prothrombin fragment 1+2 level at 0, 0.03, 0.06, 0.125, 0.25, 0.5, 1 and 2 nmol/ l (89).

3.2.6 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 platelet suspension was sonicated in iced cold water three times for 10 min. Supernatant was collected by centrifuged at 13,000 rpm for 10 min at 4 °C and stored at -20 °C until used. For the first-dimension separation, 250 µg of soluble proteins were loaded on to an Immobiline dry strip (pH 3-10 NL, with 7 cm) in a final volume of 130 µL containing 2% IPG buffer and 0.5% bromophenol blue. 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) and following a running profile as shown in Table 3. The time for the IEF step required about 8-9 hours. After the IEF process, the separation on the second-dimension step, where the IPG strips were equilibrated in equilibration buffer with 100 mM DTT added for 30 min and incubated in 125 mg iodoacetamide (IAA) for 45 min. After the equilibration step, the proteins were electrophoresed in the second-dimension using 12 % 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). Statistical analysis was performed by student's t-test with a p-value < 0.05 being considered significant.

Table 3 Isoelectric focusing running protocol

Step	Voltage mode	Voltage (V)	Duration (Volt- hours)
1	step-n-hold	300	200
2	gradient	10000	300
3	gradient	3000	4000
4	gradient	5000	4500
5	Step-n-hold	5000	3000
6	Step-n-hold	100	100

3.2.7 Tryptic in gel digestion and protein identification by LC/MS/MS

All of the differential protein spots were cut for mass spectrometry analysis. Gel pieces were washed with MilliQ water and rehydrated in 50 µL of 0.1 M NH_4HCO_3 in 50% acetonitrile. The gel pieces were destained in a solution containing 50% ACN in 100 mL of 25 mM ammonium bicarbonate (NH_4HCO_3) and then submitted to an in-gel trypsin digestion overnight at 37 °C in 25 mM (NH_4HCO_3). Peptides were extracted in 50 mL of 5% formic acid/50% ACN and then put into an ultrasonic bath for 15 min and 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 the 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).

3.2.8 Western blot analysis

Thirty milligrams of platelet protein samples were separated on 7 % polyacrylamide gel for detected integrinαIIb, 10% gel for Hsp70 and 12 % gel for PF4. 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 as shown in Table 4. After incubation, the signal was developed by adding Chemiluminescense ECL (Pierce) substrate and exposed with X-ray film.

Table 4 List of primary and secondary antibodies for western blot analysis

Antibodies	Dilution	Time (hrs)
Primary antibodies		
• Rabbit anti-PF4 polyclonal antibody	1:5000	4
• Rabbit anti-integrin αIIb monoclonal antibody	1:1000	16
• Rabbit anti-Hsp70 polyclonal antibody	1:1000	16
• Mouse anti-GAPDH monoclonal antibody	1:5000	16
Secondary antibodies		
• HRP-linked goat anti-rabbit IgG polyclonal Antibody	1:10000[a]	1
• HRP-linked goat anti-rabbit IgG polyclonal Antibody	1:5000[b]	1
• HRP-linked rabbit anti-mouse IgG polyclonal antibody	1:10000	1

a: dilution for anti-PF4

b: dilution for anti-integrinαIIb and anti-Hsp70

3.2.9 Statistical analysis

All descriptive statistics (mean and SD) of normal controls and the 2 groups of β- thalassemia/HbE patients which comprised non-splenectomized and splenectomized patients were analyzed in GraphPad Prism software version 5 (GraphPad software, La Jolla, CA). Comparison of statistical different between parameters were calculated by Student's t-test. Linear regression and Spearman's correlation coefficient (r_s) were used to analyze the correlation between parameter. Statistical values were considered as $p < 0.05$.

Chapter IV

Results

4.1 Clinical characteristics of normal controls and β- thalassemia/HbE patients

The results of laboratory parameter are shown in Table 5. Nine males and 11 females of normal controls were included, while the β- thalassemia/HbE subjects included 12 males and 11 females. The patients showed a lower red blood cell count (RBCs count), hemoglobin (Hb), hematocrit (Hct) and mean corpuscular volume (MCV), but a higher red blood cell distribution width (RDW) level compared to normal controls (*p*-value < *0.05*). There was also a significantly higher number of nucleated red blood cell (NRBCs), white blood cell count (WBC), platelet, reticulocyte and serum ferritin level in β- thalassemia/HbE patients. Furthermore, the splenectomized β-thalassemia/HbE patients showed a significantly higher number of NRBCs, WBC, platelet, reticulocyte, and serum ferritin level as compared to non-splenectomized β-thalassemia/HbE patients. However, RBCS count, Hb and Hct showed no significant difference between non-splenectomized and splenectomized β-thalassemia/HbE patients as shown in Table 6. In addition, the morphological blood pictures were assessed by Wright's Giemsa staining. The results are shown in Figure 13. The β-thalassemia/HbE patients showed increased abnormal erythrocytes with hypochromic microcytic RBCs, poikiocytosis (Schistocytes, target cells, ovalocytes, teardrop cells and anisocytocytes), and anisocytosis.

Table 5 Comparison of lab finding between normal controls and β-thalassemia/HbE patients

Laboratory parameters	Normal (n=20)	β-thalassemia/HbE patients (n=23)	Reference range	p-value
Age (years)	24-40	19-49		
Male/female (n)	9/11	12/11		
RBC x10^6 cell/ul	4.3±0.8	4.1±1.0	4.2-5.4	0.3300
Hb g/dL	14.0±1.1	7.7±1.2*	12-18	< 0.0001
Hct %	41.9±2.4	26.2±3.7*	37-52	< 0.0001
MCV fL	88.4±4.2	66.4±9.3*	80-99	< 0.0001
RDW %	12.3±0.4	22.1±2.3*	11.5-14.5	< 0.0001
NRBCs	0	133.0±64.9*	0	< 0.0001
Reticulocyte count %	0.9±0.1	5.4±4.5*	0.5-1.5	< 0.0001
WBC Count x10^3 cell/µL	5.9±0.9	8.7 ±4.3*		0.0072
Platelet count x10^3 cell/uL	289±49	343.7±231.2	140-400	0.3075
Serum ferritin (ng/uL)	76.2±37.8	979.1±701.6*	32-100	< 0.0001

* Significant difference with p-value < 0.05 compared between normal controls and β-thalassemia/HbE patients.

Table 6 Association of lab finding and splenectomy in β-thalassemia/HbE patients

Laboratory parameters	Non-splenectomy (n=15)	Splenectomy (n=8)	Reference range	p-value
Age (years)	21-49	19-41		
Male/female (n)	7/8	5/3		
RBC x10^6 cell/ul	5±1.0	3±0.4	4.2-5.4	0.0036
Hb g/dL	8.3±1.1	6.5±0.6	12-18	0.0005
Hct %	27.6±3.4	23.4±2.2	37-52	0.0059
MCV fL	62.9±9.3	72.7±5.3*	80-99	0.0138
RDW %	22.2±2.2	24.8±1.1*	11.5-14.5	0.0067
NRBCs	4.5±1.9	364.2±295.4 *	None	< 0.0001
Reticulocyte count %	2.0±0.6	11.1±1.0 *	0.5-1.5	< 0.0001
WBC Count x10^3 cell/µL	6.1±1.9	13.7±2.7*		< 0.0001
Platelet count x10^3 cell/uL	191±65	630±126 *	140-400	< 0.0001
Serum ferritin (ng/uL)	512.2±319.6	1757±382.8*	32-100	< 0.0001

* Significant difference with p-value < 0.05 compared between non-splenctomy and splenetomy β-thalassemia/HbE patients

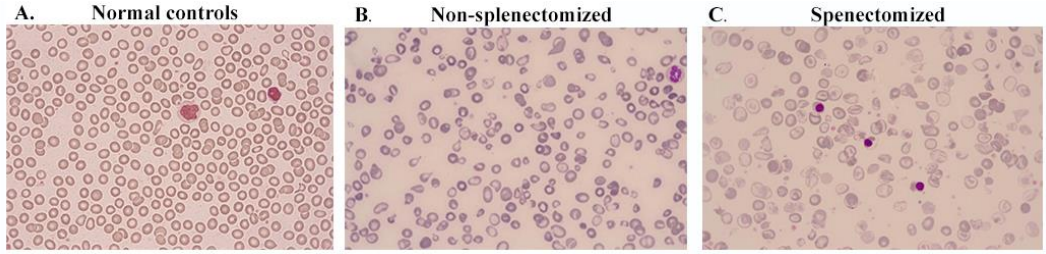


Figure 13 Peripheral blood smears of normal controls and β-thalassemia/HbE patients.

The peripheral blood smear from normal controls (A), non-splenectomized β- thalassemia/HbE patients (B) and splenectomized β- thalassemia/HbE patients (C) were stained by Wright's Giemsa staining.

4.2 Purity of isolated platelets in β-thalassemia/HbE patients

The purity of isolated platelets was determined using GPA staining and analyzed percentage of RBC vesicles as GPA-positive by flow cytometry as shown in Figure 14. The result showed that the platelet fraction of all subject groups had RBC vesicles less than 5%, including 3.9±2.0% in the splenectomized β-thalassemia/HbE patients, 0.7±0.2% in the non-splenectomized β-thalassemia/HbE patients and 0.5±0.2% in the normal control group. These results indicated that the purity of isolated platelets is high and could be used for the other experiments.

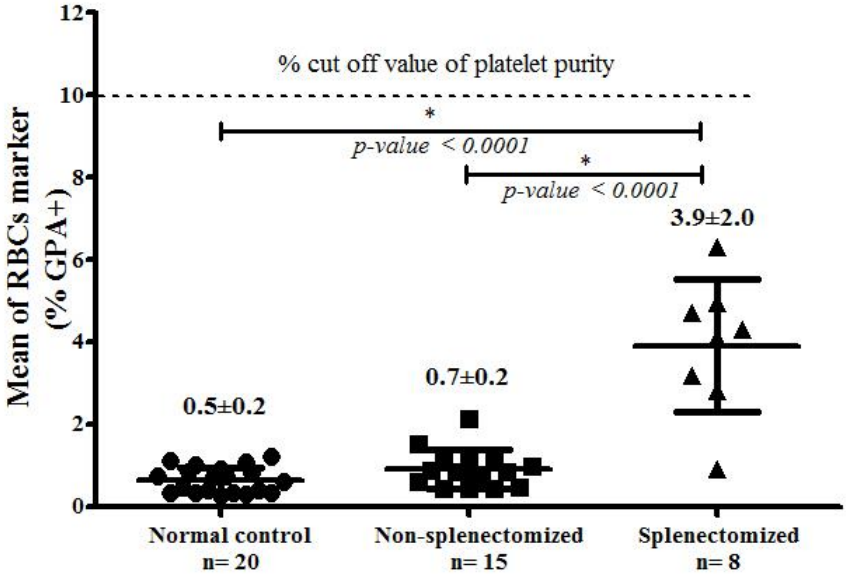


Figure 14 Percentage of RBC vesicles in isolated platelet fraction in each sample group.

The percentage of RBC vesicles in isolated platelet fraction of normal controls, non-splenectomized and splenectomized β-thalassemia/HbE patients are shown in a dot plot with dashed lines representing mean±SD, * *p*-value < 0.05.

4.3 Optimization of platelet inhibition by varying concentration of prostaglandin E1

The platelets were inhibited to stop the activation process occurring after blood collection. Prostaglandin E1 (PGE1, Clayman Chemical, Michigan, USA) was used as the platelet inhibitor in this study. The optimal concentration of PGE1 used for this experiment was tested by vary the concentration of PGE1 in 4 levels, including 1, 2, 3 and 4 µM. The percentage of platelet activation (CD62-positive) was analyzed by flow cytometry and compared to baseline of platelet activation with the result from whole blood sample. The result showed that 2µM of prostaglandin E1 is the optimal concentration which had the ability to inhibit platelet activation (Figure 15).

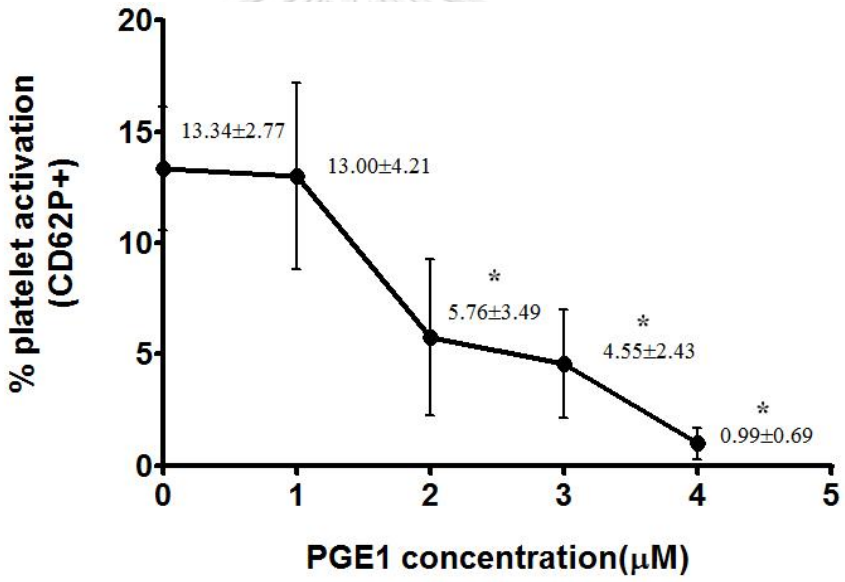


Figure 15 Percentage of platelet activation after inhibited by variable concentration of platelet inhibitor.

The platelet activation levels in isolated platelet fraction after inhibited by 1, 2, 3, 4 µM Prostaglandin E1 compared to the levels in whole blood sample. The result showed in mean ± SD and * *p*-value < 0.05.

4.4 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 (Figure 16). All samples were stained and analyzed using logarithmic amplification of forward scatter (FSC-H) and side scatter (SSC-H). The FSC-H and SSC-H scatter gram showed populations of RBCs, platelets, and MPs or debris fractions. The population of red blood cells and platelets were gated into the R1 region and R2 region, respectively (Figure 16A). Platelet population, expressed CD41-positive (R4 region), was gated from the cell population in R2 region (Figure 16B). In addition, the percentage of activated platelet (R5 region), expressed CD62P-positive, was gated from cells at R4 region (Figure 16C). 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%) (Figure 18D, E, F). 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%) as shown in Figure 17G, H, I, and J.

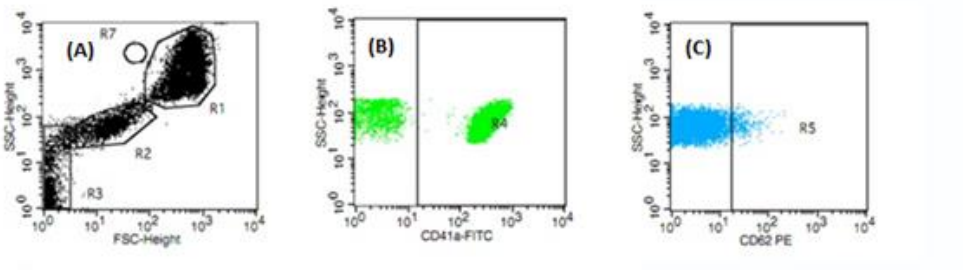


Figure 16 Platelet activation measured by flow cytometry analysis.

FSC-H/SSC-H dot plot of RBCs and platelets are shown in R1 region and R2 region, respectively (A). The CD41-positive platelets were identified in the R4 region (B). The CD41-positve platelets were analyzed for the percentage of CD62P-postive, a marker of activated platelets as shown in the R5 region (C).

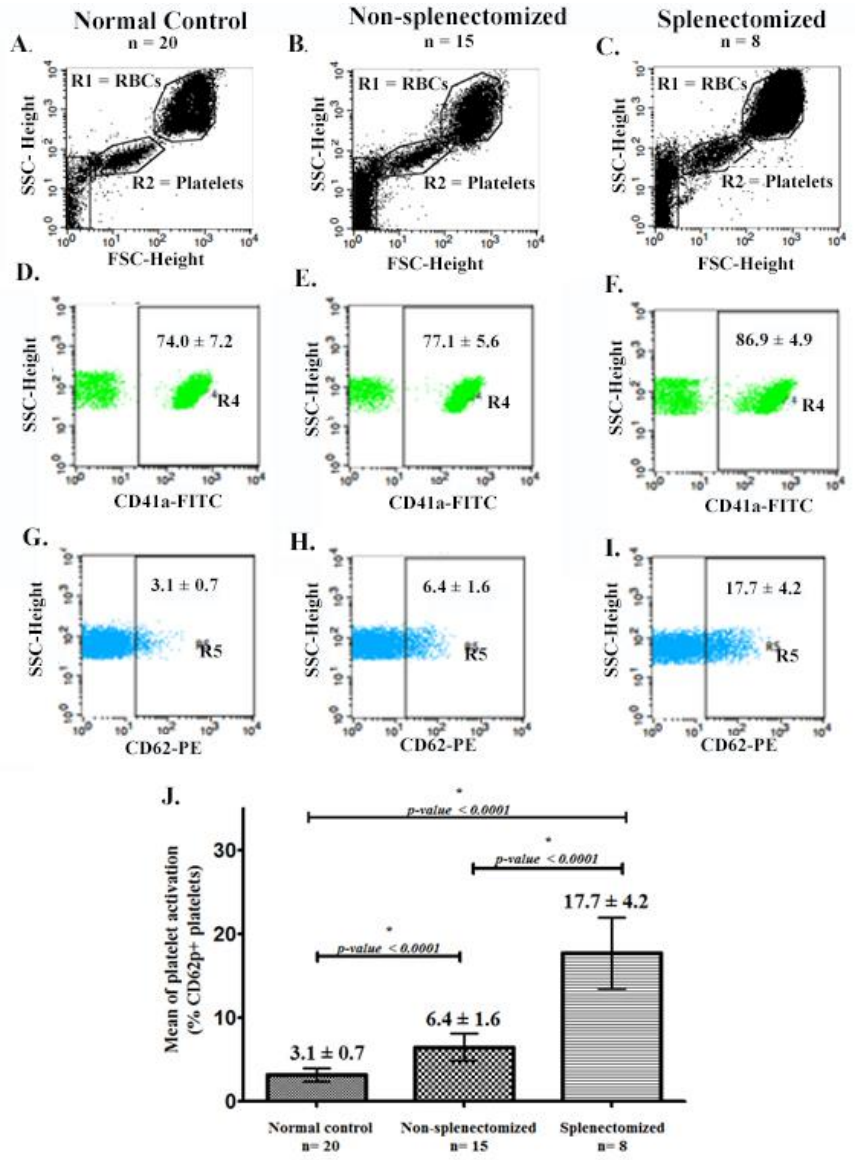


Figure 17 Levels of platelet activation in β-thalassemia/HbE patients and normal controls.

The diluted whole blood was stained with FITC-conjugated CD41 and PE-conjugated CD62P. (A) Dot plot analysis of RBCs in the R1 region and platelets in the R2 region were determined by FSC-H/SSC-H. (B) Platelets (CD 41-postive) were identified in the R4 region. (C) CD41-positive platelets were analyzed for the percentage of CD62P-positive as markers of activated platelets in the R5 region. The mean of platelet activation (The percentage of CD62P-positive) is shown in the columns with dashed lines representing mean±SD, * *p*-value < 0.05.

4.5 Increase prothrombin fragment 1+2 level in β- thalassemia/HbE patients

The standard curve of prothrombin fragment 1+2 was plotted by Curve Expert version 1.4 as shown in Figure 18. Levels of prothrombin fragment 1+2 in 8 dilutions of standard sample were measured by the Infinite Pro M200 ELISA reader (TECAN, Switzerland) and the optical densities (OD values) were added into Curve Expert software 1.4 (Figure 18A). The standard curve results were shown in Figure 18B. The OD values were added in the y-value of the analyze mode to investigate the x-value as the prothrombin fragment 1+2 concentration as shown in Figure 18C. The results showed that the prothrombin fragment 1+2 were significantly different between the groups studied as follow 0.5±0.2, 1.5±0.7 and 2.5±0.4 nMol/L for normal subjects, non-splenectomized, and splenectomized β- thalassemia/HbE patients, respectively (Figure 19). The prothrombin fragment 1+2 levels in normal controls were detected in the reference range (0.4-1.1 nMol/L). The non-splenectomized β- thalassemia/HbE patients had a three times higher prothrombin fragment 1+2 levels than normal controls. The highest prothrombin fragment 1+2 level was observed in splenectomized β-thalassemia/HbE patients when compared to non-splenectomized β-thalassemia/HbE patients and normal subjects as shown in Figure 19.

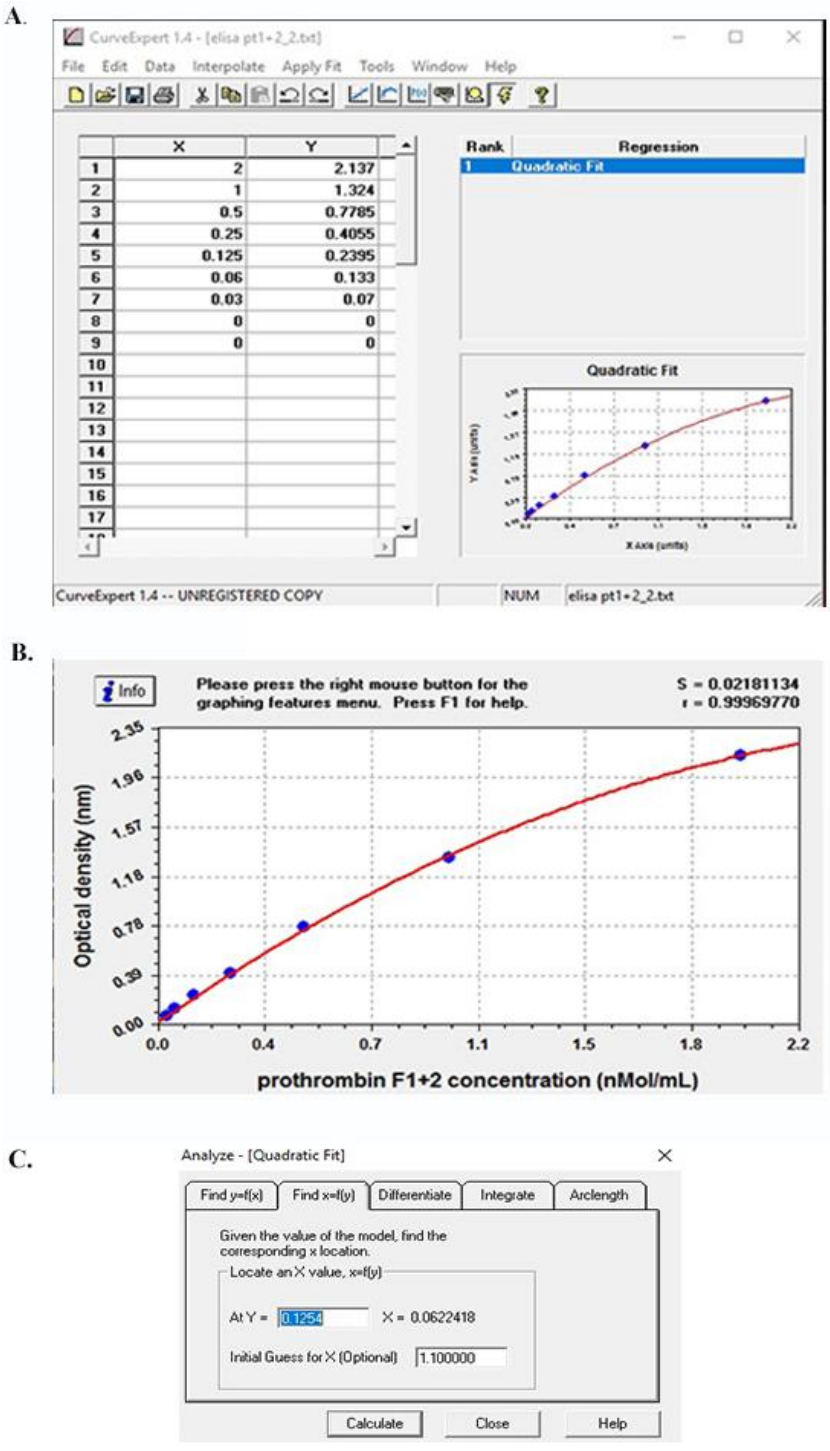


Figure 18 Standard curves of prothrombin fragment 1+2 levels.

Standard curve of prothrombin fragment 1+2 were plotted by Curve Expert software 1.4 and the prothrombin fragment 1+2 concentration of the studied samples was calculated from the standard curve.

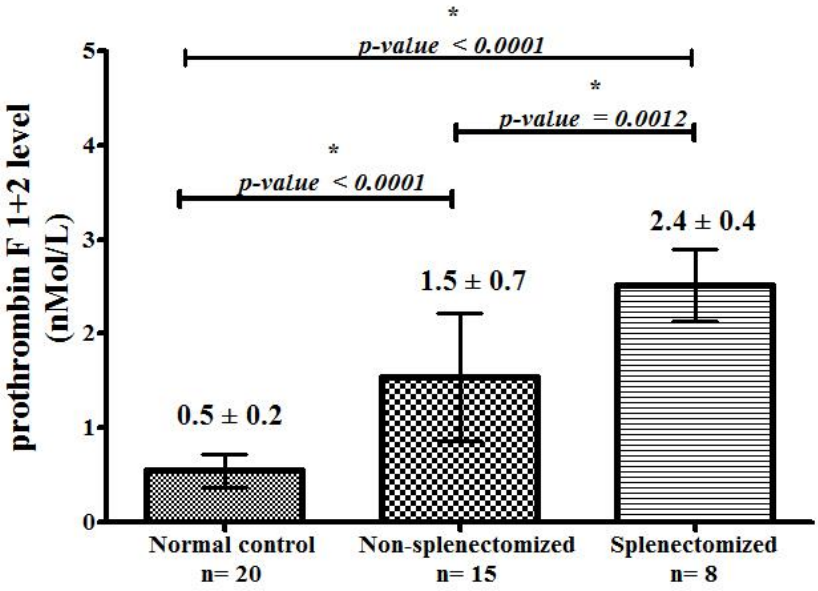


Figure 19 Levels of Prothrombin fragment 1+2 in normal controls and β-thalassemia/HbE patients.

The prothrombin fragment 1+2 levels of 8 splenectomized patients, 20 normal controls and 15 non-splenectomized patients were detected by human prothrombin fragment ELISA assay kit (A). The results of normal controls, β-thalassemia/HbE patients and the reference value are shown in the columns with dashed lines representing mean±SD (B). The statistical significance was considered at a *p*-value < 0.05.

4.6 Correlation of platelet activation and prothrombin generation product

Platelet activation and prothrombin fragment 1+2 levels represent the hypercogulation state in β-thalassemia/HbE. In this study, the platelet activation and prothrombin fragment 1+2 levels in β- thalassemia/HbE were significantly higher than normal controls. The platelet activation levels is strongly correlated with the prothrombin fragment 1+2 levels with the rs = 0.6146, p = < 0.0018 (Figure 20).

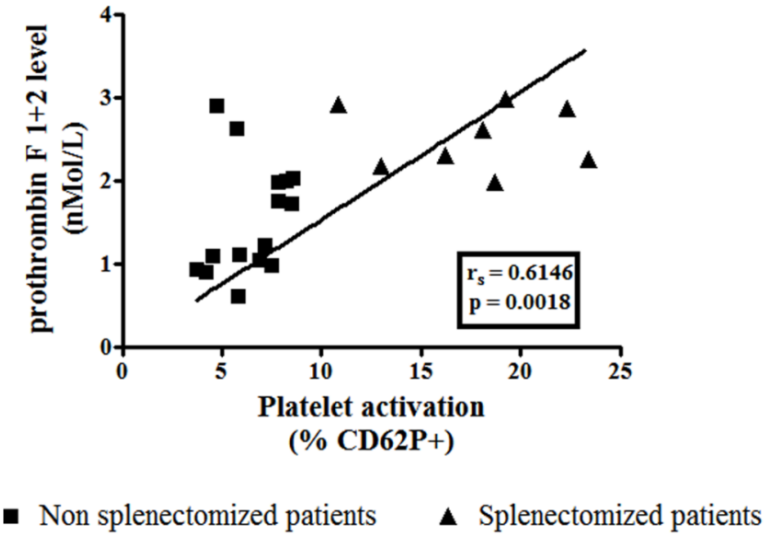


Figure 20 Correlation between platelet activation and prothrombin fragment 1+2 level.

The Spearman's rho correlation coefficient was calculated between percentage of platelet activation (CD62P-positive) and concentration of prothrombin fragment 1+2 from 20 normal subjects, 15 non-splenectomized β-thalassemia/HbE patients, and 8 splenectomized β-thalassemia/HbE patients. The statistical significance was considered at a *p*-value < 0.05.

4.7 Proteomic analysis of normal and β-thalassemia/HbE patients

Platelet proteome of 4 non-splenectomized β-thalassemia/HbE patients and 4 normal controls were analyzed by 2D gel electrophoresis. The differential protein expressions were determined by analysis of intensity, volume and area of protein spots. The results showed a total of 19 differentially regulated protein spots of which 18 were up-regulated and 1 protein was down-regulated in non-splenectomized β-thalassemia/HbE patients as compared to normal controls (Figure 21, 22). The spots were picked from the gels and subjected to tryptic digestion and LC/MS/MS analysis. The proteins were identified by searching the generated spectra against the database and showed expression of 19 platelet proteins from 19 protein spots as indicated in Table 7. The down-regulated protein was 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, 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) 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 7 Differential proteins compared 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 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
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
12	Rho GDP-dissociation inhibitor 2	23031	5.1	1229	30	1.22	-Cytoskeleton
13	Tropomyosin alpha- 1 chain	32746	4.69	134	13	1.04	-Cytoskeleton
14	Transgelin- 2	22548	8.41	3391	64	1.40	-Cytoskeleton
15	Hemoglobin subunit beta or Hemoglobin E	16102	6.75	985	58	3.90	-Globin
16	Platelet factor 4 (CXCL4 or PF4)	14171	9.04	792	45	5.42	-Chemokine -Platelet activation -Heparin binding protein
17	PDZ and LIM domain protein 1	36505	6.56	2118	48	1.02	-Cytoskeleton
18	PDZ and LIM domain protein 1	36505	6.56	2590	44	1.05	-Cytoskeleton
19	Tropomyosin alpha- 4 chain	28619	4.67	889	57	1.09	-Cytoskeleton

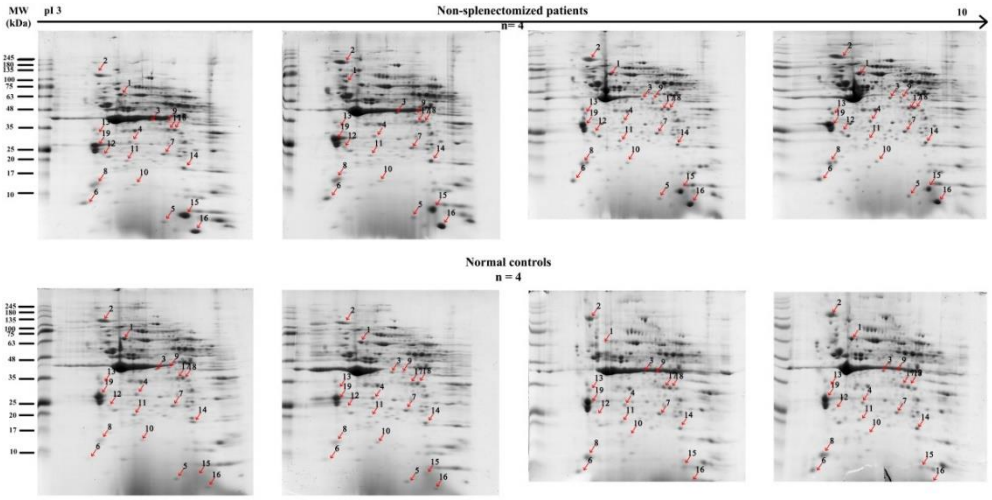


Figure 21Platelet proteome patterns compared between non-splenectomized β-thalassemia/HbE patients and normal controls.

All protein spots of 4 non-splenectomized β- thalassemia/HbE patients and 4 normal controls were detected and analyzed by Image Master 2D-Platinum v7 software (Amersham Biosciences). The red arrows showed 19 differential spots (p<0.05) when comparing between the 2 sample groups. Eighteen proteins were up-regulated and one protein was down-regulated.

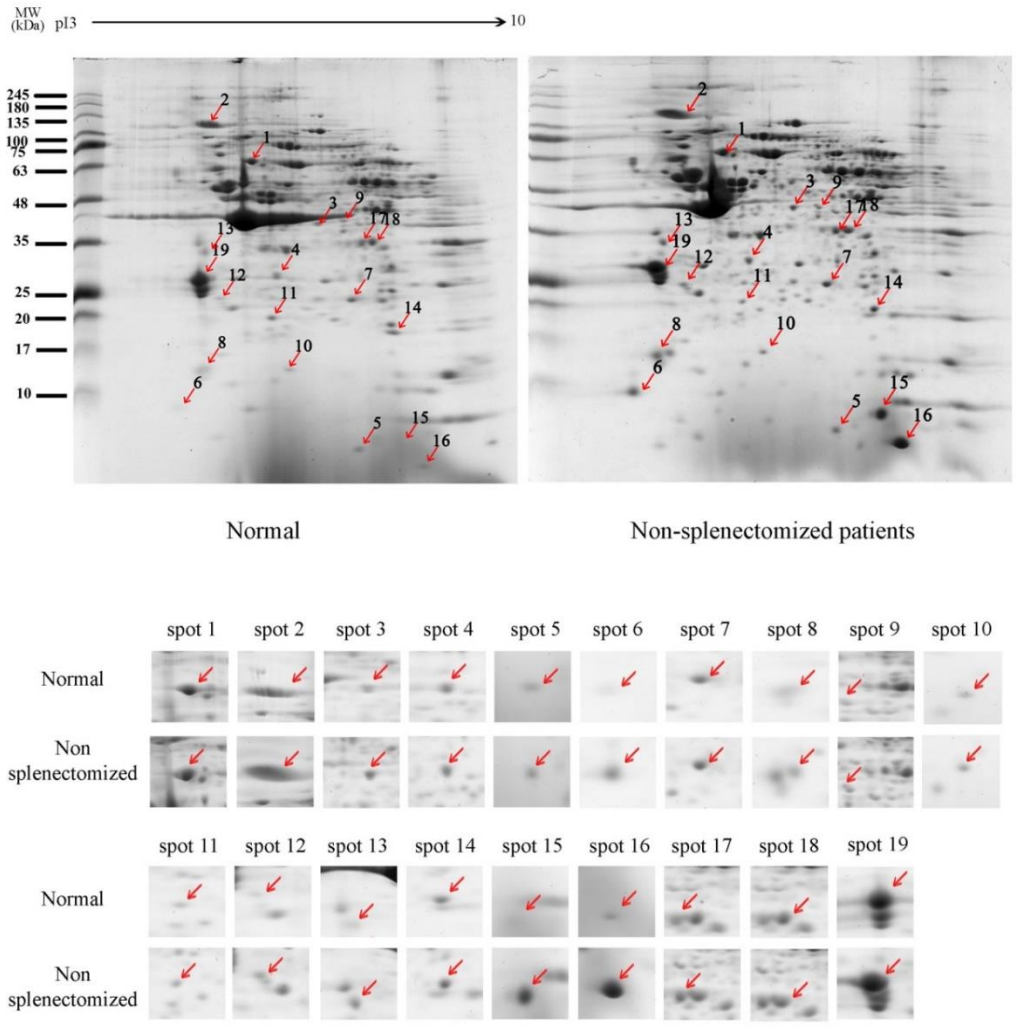


Figure 22 Enlargement of 2DE spots compared between normal controls and non-splenectomized β- thalassemia/HbE patients.

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 19 proteins (spots no. 1, 2, 4-18) were up-regulated in non-splenectomized β- thalassemia/HbE patients and one protein (spot no. 3) was down-regulated.

4.8 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. Platelet proteins were isolated from 8 normal controls, 8 non-splenectomized β- thalassemia/HbE patients, and 5 splenectomized β-thalassemia/HbE patients. After separation by SDS-PAGE, the proteins were transferred to the nitrocellulose membrane. The membranes were probes with antibodies directed to Heat shock 70 kDa protein (Hsp70), Integrin αIIb and Platelets factor 4 (PF4) and normalized by GAPDH and quantified by ImageJ version 1.8.0 (National Institute of Health, USA). 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 23.

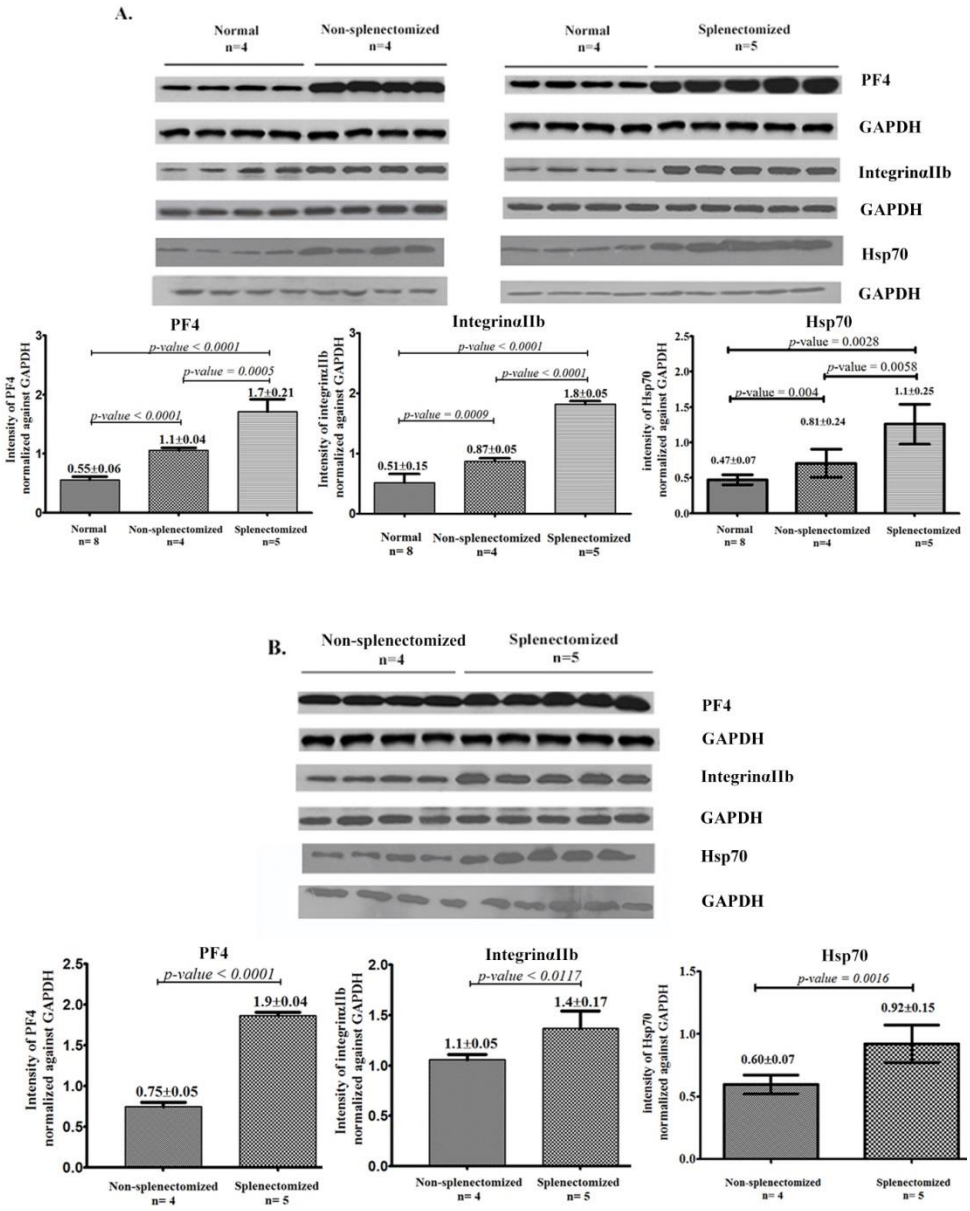


Figure 23 Expressions of PF4, IntegrinαIIb, HSP70 and GAPDH detected by Western blot analysis.

Representative Western blot analysis of PF4, IntegrinαIIb, and HSP70 against GAPDH in normal controls compared with β- thalassemia/HbE patients are shown in the upper panel. In addition, the comparison of protein expression between non-splenectomized β-thalassemia/HbE patients and splenectomized β- thalassemia/HbE patients were shown in the lower panel. Band intensities for PF4, IntegrinαIIb and Hsp70 were qualified and normalized against GAPDH.

4.9 Correlation of serum ferritin levels and expression of Hsp70, IntergrinαIIb and PF4 proteins

Correlation of serum ferritin levels and levels of 3 differentially expressed proteins including Hsp70, integrin and PF4 in β-thalassemia/HbE were analyzed. Serum ferritin levels and levels of differentially expressed proteins in β-thalassemia/HbE were significantly higher than normal controls. Expression levels of Hsp70, PF4 and IntergrinαIIb were strongly correlated with serum ferritin levels as rs = 0.80, p = 0.0009, rs = 0.75, p = 0.0032, and rs = 0.62, p = 0.024 respectively (Figure 24).

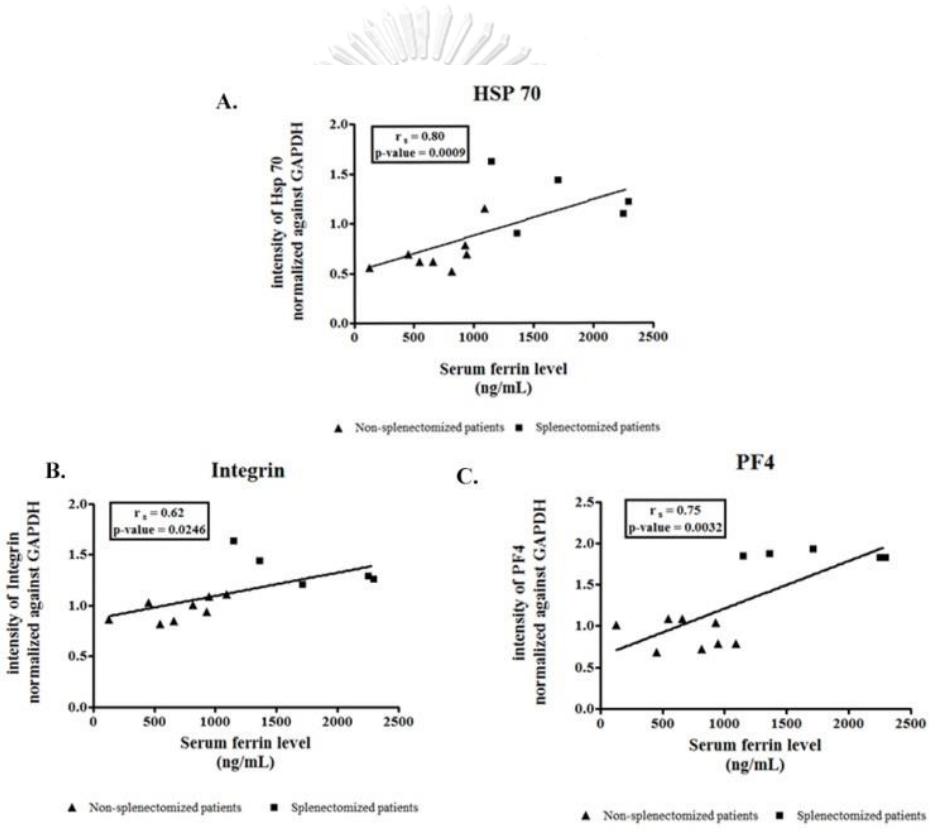


Figure 24 Correlation of serum ferritin levels and levels of differentially expressed proteins.

The Spearman's rho correlation coefficient was calculated between serum ferritin levels and levels of differentially expressed proteins including A) Hsp70 B) integrinαIIb C) PF4 from 8 non-splenectomized β-thalassemia/HbE patients, and 5 splenectomized β-thalassemia/HbE patients. The statistical significance was considered at a *p*-value < 0.05

Chapter V

Discussion

There have been many reports of increased level of platelet activation in β-thalassemia/HbE related to hypercoagulability (64, 90, 91). Previous studies suggested that hypercoagulation factor inducing hypercoagulability of β-thalassemia/HbE patients are abnormal RBCs, nitric oxide, iron overload, splenectomy and platelet activation (6, 50, 51, 92). Several studies have shown higher platelet activation levels in β-thalassemia intermedia and splencetomized thalassemia patients than in normal control (51, 93-97). In this study, the platelet activation levels were determined by flow cytometry which showed consistent data similar to the previous reports (98). We found a significant elevated platelet activation (CD41+/CD62P+) in non-splenectomized and splenectomized patients β-thalassemia/HbE patient. The increase levels of prothrombin fragment 1+2 (F1+2) which is an indicator of hypercoagulable state in β-thalassemia/HbE was also observed. The prothrombin fragment 1+2 is considered to be useful for diagnosis of thrombosis which is an activated peptide released from prothrombin during thrombin formation. Activation of prothrombin takes place in the presence of factor Xa, factor Va, calcium ions and a phospholipid surface (platelets) which is key event in coagulation of blood (99). Previous works of Atichartakarn V et al. reported hemostatic and thrombotic markers in patients with β-thalassemia/HbE patients and the result showed several hypercoagulation factors including thrombin generation as prothrombin fragment 1+2 were significantly higher in splenectomized patient and non-splenectomized patients than normal controls (100).

This study also showed that the platelet activation and hypercoagulable state are related to splenectomy which is consistent with the previous reports (51, 90, 101). These results can be explained that the increase platelet activation in β-thalassemia/HbE may cause by iron overload resulted from multiple transfusions and increased intestinal iron absorption and lower of intracellular antioxidant such as glutathione caused continuous oxidative stress leading to platelet hyperactivity (102). Moreover, the

hypercoagulable also detected in the non-splenectomized patients, indicating that these patients may also have a risk for thrombosis.

The identification of differential platelet protein expression of β-thalassemia/HbE proteomic analysis has been described from only one experiment (103). This study also used a proteomic approach to identify alterations in platelet protein levels in non-splenectomized β^0-thalassemia/HbE compared to normal controls. The results showed 19 differentially expressed platelet proteins from 19 protein spots. The proteomic analysis could not be done in the splenectomized β-thalassemia/HbE samples due to the samples had interfering in the isoelectric focusing step such as high coagulation factor concentration, salt contamination and long-term storage. However, we tested the expression of 3 differential proteins in the splenectomized patients by western analysis.

We found that most of the differential expressed proteins (10 proteins) are membrane skeleton proteins such as actin, myosin, tropomyosin and trangelin. These proteins were high abundant proteins in platelet which regulates contractile properties indicating its participation in cytoskeleton organization during platelet activation (104). Moreover, F-actin -capping protein subunit beta, Actin -related protein 2/3 complex sub unit 5, PDZ and LIM domain protein 1, tropomyosin alpha- 1 chain were up-regulated levels. These proteins were associated actin filament to polymerize with myosin proteins including myosin light polypeptide 6, myosin regulatory light chain 12A andmyosin regulatory light polypeptide 9. While actin-myosin polymerization, the elevated of tropomyosin proteins (tropomyosin alpha- 4 chain and tropomyosin alpha-1 chain) and trangelin 2 assisted actin cross-linking/gelling protein present in the cytoplasm, involved in calcium interactions in filaments in polymerization (105). Interestingly, we found the up-regulation of integrin protein, a transmembrane receptor, act as bridges between extracellular matrix to the cytoskeleton membrane resulting signaling transduction to activated platelets, platelet shape changes and platelet aggregation (106).

The up-regulation of Integrin proteins were activated by ADP, thromboxane A2 though G protein –mediated signaling pathway including Rho GDP-dissociation inhibitor 2, which also up-regulated levels in present study. This pathway activates a conformational change in the extracellular domains such as fibrinogen or von

Willebrand factor. Fibrinogens act as bridges between platelets to generate platelet aggregation (65). Additionally, we also found the up-regulation of Hsp70 which involved in platelet activation and aggregation. This result consistent with previous study, Hsp70 and integrin-αIIb were up-regulated expression in non-splenenctomized β-thalassemia/HbE patients than normal controls in 2D-PAGE and both of β-thalassemia/HbE patients were increased Hsp70 and integrin-αIIb expression levels as compare to normal controls (103).

The HSP70 associated with the inside-out activation of integrin-αIIbβ3 and activate platelet aggregation as well as granule secretion and platelet formation under physiological shear (107). These results may reflected that membrane skeletons were a first line response to stimulated platelet in shape changes step. Consequence effect of signaling transduction related to platelet integrin-αIIb and glycoprotein membrane change are releasing of its granules such as ADP (dense granules) and P-selectin and platelet factor 4 (PF4) (alpha-granules) (108). While platelet aggregation, platelet released adenosine diphosphate (ADP) which can activated platelet as agonist platelet activation (109).

Interestingly, the expression of platelet factor 4 (PF4) was a significantly higher in non-splenectomized and splenectomized patients. PF4 is a cytokine that is released from alpha-granules of activated platelets (108). PF4 plays a role in inflammation, atherosclerosis and thrombosis by neutralized heparin anticoagulant to increased formation clot stability (110-112) and heparin-induce thrombocytopenia (HIT) treatment (113). The previous study found PF4 expression in Dengue patients that involved platelet activation and platelet-mediated immune and inflammatory responses (114). However, in many studies, the plasma PF4 levels of β-thalassemia patients were measured by ELISA assay. The results showed vary results both of high and equal level when compared to normal controls (51, 115). The up-regulation of PF4 protein is a consequence effect of cytoskeleton, Hsp70 and integrin-αIIb protein changes.

Increased levels of serum ferritin were observed in this cohort. Additionally, we found the strongly positive correlation of levels of serum ferritin and Hsp70, PF4 and Integrin proteins. The serum ferritin was an iron overload marker in β-thalassemia/HbE patients that important secondary complication that occurs in β-

thalassemia resulting from red cell destruction, multiple blood transfusion and increased gastrointestinal iron absorption. The iron overload leads to accumulation of excess iron in body tissue and hemolysis of red blood cell that generated reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (116).

The ROS are suspected affect platelet function and promote platelet activation by directly; superoxide and indirectly; nitric oxides inhibition contributed to platelet aggregation and thrombus formation. The previous studies demonstrated that the Hsp70 interacted with TRiC complex and stress-induced 1 (STI1) or Hsp40 to repress heat shock transcript factor (HSF1) which increased oxidative stress responses (117).

Our result also showed the up-regulated of antioxidant protein such as peroxiredoxin 6. The elevated expression of antioxidant might be adaptive defense mechanism to counteract the increased metabolic activity and oxidative stress from iron overload. However, the present study found down-regulated levels of Leukocyte elastase inhibitor (SERPINB1) in β-thalassemia/HbE patients compared to normal controls.

The Leukocyte elastase inhibitor or SERPINB1 is an intracellular protein and a primary molecule to protect the cell from proteases reflected into the cytoplasm during stress and its role as a protector of cells from their own granular proteases (118). These results may reflect that increased free radicals in β-thalassemia/HbE patients that lead to protease inhibitor consumption and enhance apoptosis resulting in short lived platelets in patients. PF4, leukocyte elastase inhibitor and Peroxiredoxin 6 were a first report as a novel observation and require further investigations in platelet of β thalassemia/HbE patients that may improve treatment modalities for β-thalassemia/HbE patients as well as better predictors of thrombosis state in these patients.

From our result, we analyzed platelet activation represented platelet function, prothrombin fragment 1+2 represented thrombin generation and serum ferritin levels represented iron overload and the result showed splenectomized pateints had higher of these factors than non-splenectomized and normal controls. We can explain that platelet activation were associated with splenectomy status. The proteomic analysis showed 19 differential protein expression levels of platelet proteins were indicated to regulation of

platelet membrane skeleton dynamics in platelet activation which correlated oxidative stress and splenectomy status.

Platelet proteomic in β-thalassemia/ HbE patients had been done in one study in year 2016 (103). Comparing platelet proteome data between our and the previous study showed are summarized in Table 8. In contrast to the previous study, we found higher serum ferritin levels in splenectomized than non-splenectomized and normal controls. Moreover, the previous study showed higher levels of protein C which is the prothrombinase complex inhibitor in splenectomized patients than normal controls and non splenctomized patients but our study showed increase the prothrombin fragment 1+2 levels in splenectomized patients. In addition to the previous data, our study found more 14 differential proteins in patients as compared to normal controls.

In summary, the splenectomized β-thalassemia/HbE patients had a significantly increased hypercoagulation factors including platelet activation, prothrombin fragment 1+2 levels and serum ferritin levels. These results may explain that platelet activation were associated with splenectomy status by several factors in more than one way such as abnormal RBCs, PS-RBCs, micropartcles, hemolysis, iron overload and ROS resulting in chronic platelet activation and hypercoagulability in β-thalassemia/HbE patients (Figure 25). We proposed that the RBCs hemolysis products (heme, hemin and iron) were generated reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (80). The ROS are suspected affect platelet function and promote platelet activation by directly; superoxide and indirectly; nitric oxides inhibition contributed to platelet aggregation and thrombus formation (78). In other way, abnormal RBCs membranes were lost phospholipid asymmetry to expose phosphatydyl serine (PS) on their membrane and RBC derived microparticles were shed into blood circulation (119). RBCs derived microparticles, a procoagulant, can enhance prothrombin complex leading to platelet activation as well as platelet, its can produced platelet derived microparticles that activated themselves (51, 72). When platelets were activated, the platelet skeleton membranes changed and attached to extracellular matrix to secrete chemokine and aggregate to others cells such leukocyte and endothelial cells leading to formation plug (60).

Although the exact mechanism still remained unclear in splenectomized patients groups, our present study showed the highlights factors that regulated platelet

activation in establishment of hypercogulability. This result will lead some novel insight to understand the pathophysiological conditions in thalassemia patients.

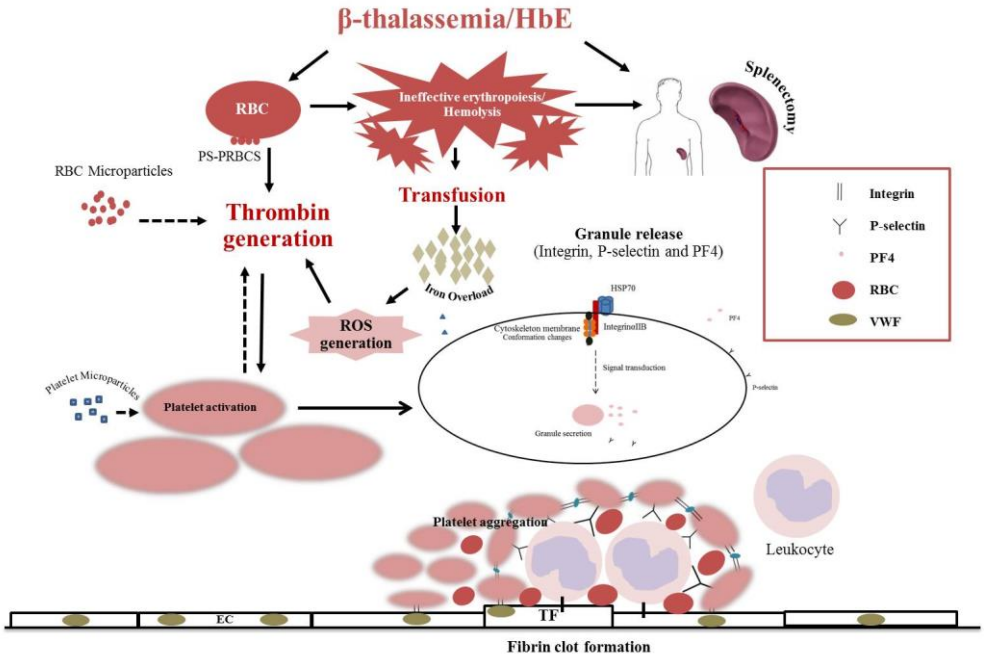


Figure 25 Schematic diagrams of hypercoagulation factors in β-thalassemia/HbE

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Table 8 Comparison of hypercoagulation factors and platelet proteomic between previous and present study

	Kamakar et al.,2016	Our study
1. Criteria of patients's blood sample	• Not clear	• Stop medicine at least 2 weeks • Stop transfusion 1 months
2. Serum ferritin levels	• Splenectomized were lower levels than non-splenectomized patients	• Splenectomized were higher levels than non-splenectomized patients
3. Platelet counts	• Non-splenectomized had lower than normal controls	• Non-splenectomized had lower than normal controls • Splenectomized had higher than normal controls and non-splenectomized patients
3. Platelet activation test	• Increased CD62P+	• Increased CD62P+
4. Coagulation levels test	• Protein C • Splenectommized had higher than non-splenectomized patients	• Prothrombin fragment 1+2 • Splenectomized were higher than non-splenectomized patients
5. Proteomic analysis	• 5 proteins (2 protein supporting data) • 2 Chaperone protein (Heat shock 70kDa protein and Protein disulfide isomerase) • 2 Antioxidant enzymes (Peroxiredoxin 2 and Superoxide dismutase) • 1 Cell survival (Translation initiation factor 5a) • 2 supporting protein (intergrin and trangelin2)	• 21 proteins • Chaperone protein (Heat shock 70kDa protein) • Fibrinogen receptor (IntegrinαIIb) • Chemokine (Platelet factor 4) • 11 Cytoskeleton proteins • 2 Antioxidant enzymes • 2 Heme synthesis proteins • 1 Intercellular energy production • 1 Immune response • 1 Protease inhibitor
6. Splenectomy status	• Data not shown	• Correlated with splenectomy status

Conclusion

1. Platelet proteomic analysis of β-thalassemia/HbE patients compared to normal controls showed a significantly increased expression of chaperone protein, integrin protein and platelet factor 4.
2. The differential protein expression levels of platelet protein were correlated with serum iron and splenectomy status.

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APPENDIX

1. Solution and buffer for proteomic analysis

1.1. Protein lysis buffer — 50 mL

(7M Urea, 2M Thiorea, 4% CAHPS, 100mM DTT,1% Protease inhibitor cocktail)

Urea	24.02	g
Thiourea	7.612	g
CHAPS	2	g

The lysis solution should be filtered through 0.2 µM and aliquot into 1 mL. 100 mL DTT and 1% protease inhibitor cocktail are freshly added before used.

1.2. 1% Bromophenol blue — 10 mL

Bromophenol blue	100	mg
Tris-base	60	mg
Deionized water (MilliQ) up to	10	mL

The solution should be filtered through 0.2 µM and aliquot into 1 mL. Store at 4 °C before used.

1.3. 30% Polyacrylamide gel, 0.8% bisacrylamide — 100 mL

Acrylamide powder	30	g
Methylene bisacrylamide	0.8	g
Deionized water (MilliQ) up to	100	mL

The solution should be filtered through 0.2 µM and aliquot into 1 mL. Store at 4 °C before used.

1.4. 10% (w/v) Sodium dodecyl sulphate (SDS) — 10 mL

Sodium dodecyl sulfate	10	g
Added distilled water to	100	mL

The solution should be filtered through 0.2 µM

1.5	**10 % (w/v) Ammonium persulfate (APS)**	**10**	**mL**
	Ammonium persulfate	1.0	g
	Added distrilled water to	10	mL

The solution should be filtered through 0.2 µM and aliwuot into 1 mL. Store at 4 °C before used.

1.6	**Tris-HCL pH 8.8**	**100**	**mL**
	Tris-base	18.15	g
	Deionized water (MilliQ)	100	mL
	Adjust pH to 8.8 with HCL		

The solution should be filtered through 0.2 µM and aliquot into 1 mL. Store at 4 °C before used.

1.7	**Equilibration buffer**	**200**	**mL**
	1 M Tris-HCL pH 8.8	10	mL
	Urea	72.08	g
	Glycerol	60	mL
	SDS	4	g
	1% Bromophenol blue	400	µL
	Deionized water (MilliQ) up to	200	mL

Mixed all of chemicals except glycerol and filtered through 0.2 µM membrane. 60 mL of glycerol was added before used.

1.8	**10X Protein running buffer**	**1000**	**mL**
	Tris	30.2	g
	Glycine	141.41	g
	SDS	10	g
	Deionized water (MilliQ) up to	1000	mL

The solution should be filtered through 0.2 µM and diluted to 1X buffer before used.

1.9	**Stock coomassie blue G 250 solution**	**1000**	**mL**
	Ammonium sulfate	100	g
	Phosphoric acid	20	mL
	Coomassie blue G250	1	g
	Deionized water (MilliQ) up to	1000	mL

Stirred solution overnight at dark condition

1.10	**Working coomassie blue G250 solution**	**1000**	**mL**
	Stock coomassie blue G250 solution	800	mL
	100% Methanol	200	mL

The solution should be freshly prepared and avoided salt precipitation during added methanol.

2. Solution and buffer for SDS-PAGE and Western blot analysis

2.1	**40% Acrylamide:bisacrylamide (49:1) stock solution**		
	Acrylamide	39.2	g
	N, N'- methylenebisacrylamide	0.8	g
	Deionized water	80	mL

The solution was dissolved at 65 °C for 10 minutes, filtered through 0.45 mm filter and adjusted volume to 100 mL with deionized water and kert at 4 °C.

2.2	**5X Sample buffer**	**50**	**mL**
	1M Tris-HCL, pH 6.8	3.125	mL
	Glycerol	10	mL
	Bromophenol blue	0.05	g
	Distrilled water	16.875	g

Added 10 µL of 1M DTT to 90 µL of 5x sample before using.

2.3	**1X transfer buffer, 10% methanol**	**1000**	**mL**
	Tris-base	1.93	g
	Glycine	9	g

	Methanol	100	mL
	MilliQ water	900	mL

Freshly prepare and precool before using.

2.4	**1X Ponceau S**	**100**	**mL**
	Powdered Ponceau S	0.2	g
	1% (v/v) Acetic acid	100	mL

2.5	**10X TBS (Tris-buffered saline) PH 7.6**	**1000**	**mL**
	NaCl	80	g
	Tris-base	24.2	g
	Distrilled water	1	L

Adjust pH to 7.6 with HCL and diluted to 1X before using.

2.6	**1X TBS, 0.05% Tween-20, 50 mM**	**1000**	**mL**
	10X TBS	100	mL
	Tween-20	0.5	mL
	Distriled water	799.5	mL

VITA

NAME: Puangpaka Chanpeng

DATE OF BIRTH: October 18, 1990

PLACE OF BIRTH: Phitsanulok, Thailand

INSTUTUTE ATTENDED: Chulalongkorn University, 2009-2012 Bachelor of Science (Medical technology)

SCHOLARSHIP: Chulalongkorn University graduate scholarship to commemorate the 72nd anniversary of His Majesty King Bhumibol Adulyadej and the 90TH anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

ADDRESS: 8/2 Thathong, Meung District, Phitsanulok, Thailand 65000

PRESENTATION

1. Poster presentation

Puangpaka Chanpeng, Kamonlak Leecharoenkiat. Increased platelet HSP70 protein is related to iron overload in β-thalassemia/ HbE disease. The 8th National and International Graduate Study Conference) (Thailand 4.0 Creative Innovation for Sustainable Development). 28-29 June 2018.

2. Proceeding presentation

Puangpaka Chanpeng, Saowaros Svasti, Duncan R. Smith, Kittiphong Paiboonsukwong, Wasinee Kheansaard, Wannapa Sornjai and Kamonlak Leecharoenkiat. Platelet proteome and hypercoagulable state of non-Splenectomized β-Thalassmia/HbE patients. ICKSH 2018 (2018 Korean society of hematology international conference& 59th Annual meeting). 29-31 March 2018.