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Proteomic Analysis of β -Thalassemia/HbE: A Perspective from Hematopoietic Stem Cells (HSCs)

Saranyoo Ponnikorn, Siripath Peter Kong, Sasipim Thitivirachawat, Chanawin Tanjasiri, Sumalee Tungpradabkul and Suradej Hongeng

Abstract

β -Thalassemia/HbE is highly prevalent in Southeast Asian countries, especially Thailand. It is a severe hereditary anemia disease involving ineffective erythropoiesis in the bone marrow and peripheral tissues. The excess of alpha globin and iron overload contribute to elevated oxidative damages leading to a premature cell death of erythroid cells and a diminished terminal differentiation of reticulocytes. Although proteomic approach would gain a comprehensive picture of the complex pathophysiology of human bone marrow and hematopoietic stem cells (HSCs), obtaining sufficient clinical specimens remains an important issue. The employment of mass spectrometry (MS)-based proteomic profiling could overcome these constraints and provide useful insights into the cellular constituents and microenvironment in bone marrow milieu. In this chapter, we summarize the comparative proteomic studies analyzing CD34⁺/HSCs and bone marrow niche proteins. Under ineffective erythropoiesis, in-depth analyses of various proteome profiles revealed many of which have putative functions. Importantly, dysregulated cell death and survival signaling pathways could explain the deleterious pathogenesis of β -thalassemia/HbE.

Keywords: β -thalassemia/HbE, hematopoietic stem cells (HSCs), ineffective erythropoiesis and proteomics

1. Introduction

Erythrocytes contain the hemoglobin (Hb) protein that carries oxygen from the lungs to the rest of the body. During the development, the hemoglobin structure gradually changes. The fetal hemoglobin HbF ($\alpha_2\gamma_2$) is a major hemoglobin in the human fetus during the last 7 months of development in the uterus. The adult hemoglobin contains two components, the major hemoglobin, Hb A ($\alpha_2\beta_2$), and the minor hemoglobin, Hb A2 ($\alpha_2\delta_2$) [1, 2]. The composition of heme and heterodimeric forms of globin protein, α -globin and β -globin, is encoded by the α gene and the β gene, which are in chromosome 16 and 11, respectively. The inherited

hemoglobin disorder is a common monogenic disease that could be identified into two main groups, the hemoglobinopathy which is abnormal structure hemoglobin variants and the recessive inheritance allele which is the defective hemoglobin production, the thalassemia syndromes [3, 4]. More than 200 point mutations and 80 different deletions within the β -globin-encoding gene are related to inherited hemoglobin disorder of β -thalassemia that represent in population worldwide [5]. In Southeast Asia, especially Thailand, hemoglobin E (HbE) thalassemia accounts for about one-half of all cases, with the highest frequencies observed. Approximately 35,000 patients are living with β -thalassemia syndrome in Thailand, 7% with a β -thalassemia trait, and 17% with an HbE trait within a population of 65 millions of Thai people [6].

Pathophysiology of β -thalassemia/HbE is strongly associated with the excess of unmatched α -globin chain and mutation of hemoglobin variants which are apparently observed inside the bone marrow, as well as peripheral tissues including the liver and spleen [7, 8]. These phenomena are believed to be the etiological factors of ineffective erythropoiesis. However, these conditions are found in certain hereditary blood disorders and are often associated with β -thalassemia/HbE [9]. This disease is characterized by apoptosis of erythroid precursors and inhibited erythroid differentiation and maturation [10]. These lead to an insufficient number of red blood cells. Consequently, a decreased oxygen-carrying capacity of the blood is tightly linked to the compensatory mechanism of the elevated erythropoietin (EPO) production that later causes the deleterious effects [11].

Erythropoiesis is a complex orchestrated process involving self-renewal, proliferation, differentiation, and mobilization of HSCs that gives rise to erythroid lineages [12]. These processes are governed by heterogeneous regulatory factors, cytokines, signaling molecules, and various cellular interactions. Erythropoiesis is extensively studied in mice model, although there are several species-specific differences of erythroid differentiation [13]. Many *in vitro* studies focused on cultures of human hematopoietic stem cells (HSCs), and erythroid progenitor cells have been elucidated the pathogenesis of β -thalassemia/HbE [14–20]. However, studying of homeostatic imbalance inside the bone marrow regarding ineffective erythropoiesis is challenged by limited human samples.

The emergence of “OMIC” approach, especially proteomics, has accelerated our understanding of pathobiology of the β -thalassemia/HbE disease, which will have a substantial impact in medicine. Technological advances of proteomic analysis tools, for example, mass spectrometry, allow high sensitivity, reduced sample requirement, increased throughput, and dissect posttranslational modifications of limited clinical specimens [21, 22]. The use of these technologies continues to expand substantially, particularly to meet the need for better diagnostics and to shorten timeline of effective therapy development. Interestingly, in combination with optimized *ex vivo* HSCs to erythroid culture or freshly isolated samples, MS-based proteomic approach has revealed a more comprehensive understanding of ineffective erythropoiesis in β -thalassemia/HbE.

2. Pathophysiology of β -thalassemia/HbE

β -Thalassemia is characterized by insufficient β -globin chain production due to decreased production (β^+) or null production (β^0) of β -globin chain, which is the main component of adult hemoglobin. This condition leads to variable clinical symptoms. β -Thalassemia major is characterized by severe anemia. Individuals with homozygous β -thalassemia may develop β -thalassemia major or β -thalassemia intermedia. The concentration of hemoglobin could distinguish types of β -thalassemia

[3, 4, 23, 24]. β -Thalassemia major has low levels of hemoglobin of 3–4 g/dL, whereas the mild-to-moderate anemia show hemoglobin concentration at 7–10 g/dL. Clinical complications of β -thalassemia major include growth retardation, pallor, jaundice, poor musculature, genu valgum, hepatosplenomegaly, and leg ulcers. A severe anemia is accompanied by ineffective erythropoiesis with bone expansion and extramedullary erythropoiesis in the liver, spleen, and other sites such as paravertebral masses. These lead to the skeletal changes including deformities in the long bones of the legs and typical craniofacial changes [3, 25]. Typical treatment for β -thalassemia major is primarily blood transfusion which causes another complication, posttransfusional iron overload. In some cases, chelation therapy could be implemented.

β -Thalassemia/HbE has extremely diverse clinical phenotypes because of various genetic lesions of the gene affecting different impaired globin chain synthesis and abnormal forms of hemoglobin variants [3, 4]. The genetic mutation of the β -globin-encoding gene at codon 26 (GAG to AAG) contributes to the activation of cryptic splice site that cause abnormal mRNA processing. The β^E -chain are produced at a reduced level resulting in a mild β -thalassemic phenotype [26]. Each year in Thailand, approximately 3000 infants are born with β -thalassemia/HbE either a mild β -thalassemia or a severe transfusion-requiring β -thalassemia major. HbE/ β -thalassemia patients have remarkable variation in terms of severity reflecting the heterogeneity of the β -thalassemic mutations in the HbE gene and other modulation factors. Newborns carrying HbE/ β -thalassemia mutations are usually asymptomatic because they rely on the HbF hemoglobin rather than the HbE. After 6–12 month of birth, anemia with abdominal enlargement due to hepatosplenomegaly could be observed [27–30]. Although the defect in β -globin production could be explained by the mutations or the absence of the β -globin gene or other modification factors, the variable severity of β -thalassemia is also involved in an imbalance of β - and α -chains, particularly in maturing erythroid cells [31]. The accumulating unmatched free α -globin chain is unstable and could precipitate in the erythroid precursors in the bone marrow as well as in peripheral blood. A severe accumulation of the α -chain forms hemichromes which cause uncontrollable oxidative damages in erythroid precursor and erythroid cells. The hemichromes can generate reactive oxygen species (ROS) that potentially damage important proteins, DNA, and other cellular components and trigger signaling cascades of programmed cell death [10, 32–36]. The destruction of erythroid cells is an onset of dramatic amelioration cycles inside the bone marrow and peripheral organ due to the ineffective erythropoiesis in β -thalassemia/HbE patients.

3. Ineffective erythropoiesis

Erythropoiesis is a progressive orchestrated process involving heterogeneous regulatory factors such as cytokines, signaling molecules, and various cellular interactions which play important roles in self-renewal, proliferation, differentiation, and mobilization of HSCs to become erythroid lineage [13, 37]. The ineffective erythropoiesis is the hallmark pathophysiological condition having seen in β -thalassemia/HbE. In β -thalassemia/HbE, the oxidative damage caused by the accumulation of hemichromes and the generation of ROS in red blood cells (RBCs) and erythroid progenitors are an etiologic factor perturbing the homeostasis of erythropoiesis [8]. In vitro and in vivo studies of mice model and ex vivo studies of human HSCs and erythroid progenitors have elucidated the molecular pathogenesis of the ineffective erythropoiesis of β -thalassemia. The excess of α -chain is believed to accelerate apoptotic cell death in erythroid precursors [10], while the inhibited processes involving

differentiation of erythroid maturation have been observed during polychromatophilic stage [34]. Both detrimental processes lead to an insufficient number of RBCs and decreased oxygen-carrying capacity of blood and ultimately cause hypoxia condition. The lack of mature RBCs stimulates a compensatory mechanism of erythropoiesis that triggers the overproduction of EPO from the kidney. The increased EPO could induce erythropoiesis in the bone marrow by inducing proliferation of HSCs and erythroid progenitors. The EPO also triggers survival mechanism under stress condition and supports differentiation of mature RBCs. However, the dramatic oxidative damages inside the bone marrow are the key factor that generates a homeostatic imbalance of erythropoiesis. This is linked to an erythroid expansion leading to extramedullary hematopoiesis [37]. Dysregulated hemoglobin synthesis and hemolysis are major factors of the ineffective erythropoiesis and determine the severity of the disease [38]. Another clinical complication is an iron overload in severe patients that are treated by regular blood transfusions which is not required for thalassemia intermedia patients. Blood transfused patients usually develop elevated body iron load due to an increased gastrointestinal iron absorption. Consequently, the accumulation of iron also causes oxidative stress because they produce highly reactive hydroxyl radicals that could not be naturally degraded by enzymatic reaction. Altogether, the accumulation of unstable free α -globin, heme, and iron all contributes to destruction of cellular compartments in the bone marrow of β -thalassemia patients [7, 39, 40]. Previous studies attempted to identify molecular and cellular mechanisms associated with the ineffective erythropoiesis in β -thalassemia patients. However, the bone marrow is tightly linked to other vital organs and complicates the deciphering of ineffective erythropoiesis of β -thalassemia.

Although there are well-established genetic manipulation techniques in model organisms such as mice and zebrafish and extensive enrichment methods of specific cell stage of hematopoietic progenitors make functional study of hematopoiesis possible, many components of the process act differently in human due to the evolutionary distance of hematopoietic genes, transcription, and epigenetic regulations [13]. *Ex vivo* human model using primary cell cultures of HSCs obtained from the peripheral blood, cord blood, and bone marrow or fetal liver of donor remains a gold standard of erythropoiesis study. Notably, involving multifactors could be determined in very proliferated and differentiated cells in different culture systems [41]. However, in expansion and differentiation steps, primary cell culture system may not provide sufficient amounts of analytes for some downstream techniques such as Western blot. While most techniques have their limitation to systematically analyze the bone marrow, its microenvironment and other neighboring cellular interactions during pathological conditions of ineffective erythropoiesis of β -thalassemia/HbE. Alternative approaches could be implemented to analyze the limited isolated primary cells of thalassemia patients and potentially other dys-erythropoietic diseases. Here, we discuss advantages of OMIC approaches especially proteomics for studying molecular pathogenesis of β -thalassemia/HbE.

4. Clinical proteomics of β -thalassemia/HbE

One of the goals of proteomic study is to characterize the flow of information through complex protein networks in a particular disease model, where the changes of proteins in the whole proteome can be identified in the pathophysiological conditions at different disease developmental stages. Comparative proteome profiling of patient and healthy subjects may help to provide new clinical conceptualization for systemic pathophysiology including root causes, biomarkers, and applicable treatment regimes. Clinical proteomics is an exciting sub-discipline of proteomics

that will integrate the application of proteomic technology to identify problems at the bedside [42]. Conventional analysis methods, such as Western blot, are difficult because of limited amounts of clinical samples in many disease models. In the field of biomedical research, nonetheless, the cause of the disease could not be thoroughly explained due to the complexity of various molecular and cellular pathways in many types of cells and tissues. Therefore, elucidation of protein network alteration in clinical samples is an advantage strategy for deciphering interaction between proteins and their expression profiles which are related to cellular physiology [43]. Despite high-throughput genomic technology can identify differential gene expression in disease samples, proteome alterations still occur in many ways not predictable from genomic analysis. A better understanding of these alterations will have a substantial impact in medicine [44, 45]. Moreover, clinical proteomic is a very promising tool for identifying various posttranslational modifications, such as phosphorylation and glycosylation. The recent advance proteomic analysis provides high sensitivity, reduced sample requirement, increased throughput, and is able to uncover various posttranslational modifications which are available for disease-related applications [46]. The use of these technologies will likely expand substantially, particularly to meet the need for better diagnostics and to shorten the path for developing effective therapy.

Proteomic analysis of blood samples has become an interesting method in hematological studies. The extremely rich spectrum of proteins in blood and blood components serves various cellular activities including coagulation, transport, immune system and cell signaling, as well as by-products of cellular damage and proteins from other tissues [47]. Whereas, the identification of originality related hematopoiesis from HSCs in some clinical respects such as leukemia and anemia are very challenging [48]. Since the key candidate proteins belonging to any disease status are in the low-abundance level, to overcome these problems, protein samples are fractionated to allow effective detection and quantification by mass spectrometry (MS) [21]. Different protein separation methods influence MS results. Many previous clinical proteomic studies of β -thalassemia/HbE employed gel-based technique and shotgun proteomic to analyze posttranslational modifications of the thalassaemic proteome. Most comparative protein profiles of β -thalassemia/HbE between patients with normal subjects from various types of clinical specimens including plasma serum, platelets, platelet-free plasma-derived microvesicles, and circulating extracellular vesicles (EVs) have been reported [49, 50]. Interestingly, a few studies have directly investigated proteomic changes inside the bone marrow or isolated the HSCs of thalassaemic samples, where are the major site of erythropoiesis and could identify relevant proteins in the ineffective erythropoiesis. HSCs were isolated from bone marrow cells and peripheral blood using antibody specific to CD34, the transmembrane glycoprotein. Both peripheral blood- and bone marrow-derived CD34⁺/HSCs showed differentially regulated proteomes during ineffective erythropoiesis in β -thalassemia/HbE [15, 18]. Recently reports have revealed the nature of the bone marrow plasma proteome and the roles of microenvironment in the bone marrow niche and linked to the dyserythropoiesis of β -thalassemia/HbE [51].

4.1 Gel-based technique

Gel-based proteome analysis method involves a separation of proteins using either one-dimensional (1-D) or two-dimensional electrophoresis (2-DE). Since 2-DE separates proteins by their isoelectric points and by molecular weight, it is a standard choice for protein separation. After the proteins are resolved, the gel is visualized by staining with visible dyes, such as Coomassie Blue or silver nitrate or with fluorescent dyes such as Sypro Ruby. The gel is imaged and analyzed by

software to identify protein spot that show altered intensity. Selected protein spots are excised and subjected to in-gel digestion typically by trypsin, resulting in peptides that can be further analyzed by mass spectrometry (MS) or tandem mass spectrometry (MS/MS). One of the drawbacks of the conventional 2-DE is gel-to-gel variability. For many proteomic studies, multiple gels of many samples have to be run, and spots at a certain location have to match between gels to reduce variability, but this still cannot fully eliminate the problem. Apart from variability, 2-DE also has poor reproducibility and provides relatively lower sensitivity. To circumvent these problems, two-dimensional difference gel electrophoresis (2-DIGE) has been developed. By labeling different samples with different fluorescent dyes and separating them in the same gel, 2-DIGE enables better comparison of up to three protein samples [22, 52, 53]. However, there are some proteins cannot be resolved on 2-D gels due to their physicochemical properties. These include highly basic or acidic proteins, proteins with extremely high or low molecular weight, and membrane proteins that are inherently insoluble in gel matrices [21].

Two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry or tandem mass spectrometry (MS and MS/MS) has been used for the investigation of β -thalassemic proteomes. The majority of differential expressed proteins between β -thalassemia patients and normal clinical specimens have been identified and characterized their functions in different metabolic processes in response to inorganic substances using an online DAVID gene ontological analysis [54]. Several enzymes in metabolic processes are usually abundant and easily found on the 2-DE, whereas low-abundant proteins involve in gene regulation and signal transduction cascades are difficult to detect. However, 1D-SDS-PAGE may be used as a standard separation method for complex protein mixtures based on their molecular weight prior MS analysis. Gel-enhanced liquid chromatography coupled with tandem mass spectrometry (GeLC-MS/MS) is a direct intensive methodology that provides high coverage of the proteome. GeLC-MS/MS is applicable to reduce gel-to-gel variation and inconsistency of protein spots in a 2-DE and to increase sensitivity of detection of low-abundant proteins [55]. Current study of bone marrow plasma proteome profile using GeLC-MS/MS technique has reported differentially expressed proteins between patients and normal donors and showed that many of which have functions in intracellular signaling and metabolic pathways associated with ineffective erythropoiesis [51]. GeLC-MS/MS has been used to analyze the CD34⁺/HSCs' thallemic proteome during the in vitro differentiation of erythroid lineages and identified dysregulation of many signaling protein of differentiation processes in thallemia patients (unpublished data).

4.2 LC-based technique

LC-based technique or shotgun proteomic analysis is an advanced and widely used approach for protein identification and characterization of sequence variants and posttranslational modifications [56]. Shotgun proteomics is considered as a bottom-up approach, by which short peptides are prepared by proteolytic digestion, usually by trypsin, and subjected to protein sequencing via tandem mass spectrometry (MS/MS) and automated protein database searching. After proteolytic digestion by trypsin, short peptide mixture is fractionated by hydrophobicity and charged using multidimensional liquid chromatography. Eluted peptides from the column are then ionized and separated by m/z in the first stage of MS/MS. Fragmentation is initiated when the peptide ions undergo collision-induced dissociation (CID) within inert gas. The ionized fragments are then separated by m/z in the second stage of MS/MS, and the corresponding peptide ions generate fragment ion spectral fingerprints for peptide identification. The process continues

until all peptides are eluted from the chromatography column. Peptide identification is accomplished by comparing the fragmentation spectra derived from peptide fragmentation with theoretical MS/MS spectra based on in silico protein database. Protein inference is achieved by allocating peptide sequences to proteins [56–58]. Shotgun proteomics has a relatively high throughput compared to other MS-based proteomic technologies. This gel-free approach involves proteolytic digestion of proteins into short peptides (1–3 kDa) as they are easier to fragment than intact proteins simplifying MS/MS sequencing [21].

Erythropoiesis involves posttranslational modifications of proteins associated with cell-cell communications and cell interactions inside the bone marrow. Phosphorylation is mediated by kinase enzymes which have different target specificities. Different phosphorylation sites on the same protein usually serve different protein activities. Shotgun proteomic strategy becomes the widely used for studying phosphoproteomes. The CD34⁺/HSCs' isolation from the bone marrow is a good representative model for elucidation signaling proteins using phosphoproteomic analysis comparing with thalassemia patient and normal donor. The workflow includes an enrichment of phosphoproteins among patient and donor samples using immobilized metal affinity chromatography (IMAC), followed by ESI-LC-MS/MS. This approach detects upregulation of signaling proteins of apoptosis pathway linked to ineffective erythropoiesis in β -thalassemic CD34⁺/HSCs [15].

5. Alteration of proteome profiles in hematopoietic stem cells (HSCs) derived from β -thalassemia/HbE patients

The hallmarks of stem cells include self-renewal and differentiation ability to give rise different types of mature cells. Stem cell biology is an important field of biomedical science and holds promise in clinical applications. The hematopoietic stem cells (HSCs) are multipotent progenitor cells located in adult bone marrow, responsible for blood cell production. Multipotency or multi-lineage differentiation potential of the HSCs defines as the capacity to give rise to several differentiated cell types including myeloid progenitor cells, erythroid-megakaryocyte progenitor cells, and lymphoid progenitor cells [13]. In addition, the proliferation and differentiation of HSCs into the erythroid lineage are the acquisition of functional and structural properties associated with erythrocyte physiology [37, 59]. The cell isolation technique of the primitive HSCs' population expressing specific cell surface markers, CD34 and CD133, is a gold standard method widely used for investigating of erythropoiesis in vivo [60]. Using antibody-conjugated magnetic particles is the first step after the isolation of mononuclear cells from bone marrow aspirate or from peripheral blood. Optimized culture condition for in vivo erythroid development requires defined medium supplemented with cytokines, such as erythropoietin (EPO), to modulate erythroid cell development from HSCs/CD34⁺. Erythroid differentiation is a multistep process starting when a subset of HSCs/CD34⁺ commits to differentiate to the megakaryocyte-erythrocyte progenitors (MEPs). The differentiation of MEPs into the most immature erythroid progenitors, namely, a burst-forming unit erythroid (BFU-Es), involves a number of transcriptional regulators. The BFU-Es generate colony-forming units-erythroid cells (CFU-Es) which strictly depends on the erythropoietin to induce differentiation via erythropoietin receptor (EPOR). Subsequently, the CFU-Es develop into proerythroblasts (the first erythroid precursors) through the basophilic, the polychromatophilic, and the orthochromatic stages. During this differentiation process, the hemoglobin synthesis, cellular content modification, and nuclear condensation gradually take place, followed by the enucleation step generating reticulocytes which are immature red

blood cells (RBCs). In addition to the macrophage-induced cellular death pathways, autophagy is the key mechanism of the biconcave formation of mature RBCs [13]. Conversely, in β -thalassemia/HbE patients, the ineffective erythropoiesis has dramatic effects in the erythroid lineage development, resulting in hyperproliferation, increased apoptosis, and inhibited terminal erythroid differentiation. To study the pathogenesis of β -thalassemia/HbE, our strategies include the isolation of CD34⁺/HSCs, in vitro erythroid differentiation, and bone marrow plasma enrichment followed by the MS-based analysis to comprehensively understand the early molecular basis of β -thalassemia/HbE. This method revealed the bona fide proteome alterations of ineffective erythropoiesis from CD34⁺/HSCs, the dynamic differentiation of CD34⁺/HSCs in the erythroid lineage, and bone marrow microenvironment (**Figure 1A**). The bioinformatic approach has also been applied to analyze integrated thalassemia-specific proteomes in a range of patient specimens. We report interesting imbalance signaling landscapes which may contribute to ineffective erythropoiesis found in β -thalassemia/HbE patients.

5.1 Metabolic enzymes

Leecharoenkiat et al. reported the first differential 2-DE proteome between β^0 -thalassemia/HbE and control erythroblasts isolated from peripheral CD34⁺/HSCs. Many differentially expressed proteins identified in this study were constituents of the glycolysis and TCA pathways and were correlated with the degree of erythroid expansion [18]. Our investigation of bone marrow microenvironment proteome has shown increased level of ATP citrate lyase (ACLY) in β -thalassemia patients. ACLY is primarily responsible for synthesis of acetyl CoA in TCA cycle. Consequentially, hyperproliferation of erythroblast during ineffective erythropoiesis might be related with the increase of ACLY indicating a high metabolic flux governed by acetyl-CoA production. ACLY can support sufficient energy to drive the TCA cycle and mitochondrial oxidative phosphorylation [51]. Therefore, the comparative proteome profiling of peripheral blood CD34⁺/HSCs and bone marrow plasma proteome indicated the similar target metabolic protein biomarkers associated with ineffective erythropoiesis.

5.2 Oxidative damage and antioxidant proteins

An imbalance between oxidative damage and antioxidant enzymes is associated with cellular pathology inside HSCs and differentiated erythroid precursors in β -thalassemia patients. Comparative proteome analysis during differentiation of CD34⁺/HSCs through erythroid lineage for 7 days in β -thalassemia patients compared with normal showed many proteins differentially expressed; these include superoxide dismutase (SOD), peroxiredoxin 6 (PXD6), and peroxiredoxin 2 (PXD2). An increased level of PXD2 was found in differentiated thalassemic CD34⁺/HSCs at day 0 through day 7 (unpublished data). In addition, PXD2 has function in stress response in the protective system during stress erythropoiesis in thalassemic mice model [61]. PXD2 is an abundant protein mostly identified in many proteomic studies from various clinical specimens of β -thalassemia [50, 54, 62]. An increased oxidative damage from alpha excess and iron overload thus triggers the cellular defensive mechanism, including antioxidative responses. Nevertheless, thalassemic stem cells could not prolong survival pathways to prevent cell death. Moreover, comparative bone marrow plasma proteome from patients and normal showed a decreased level of an antioxidant secretory or extracellular exosomal protein, namely, apolipoprotein D (ApoD), in patients who have an impairment of bone marrow microenvironment to dyserythropoietic condition [51].

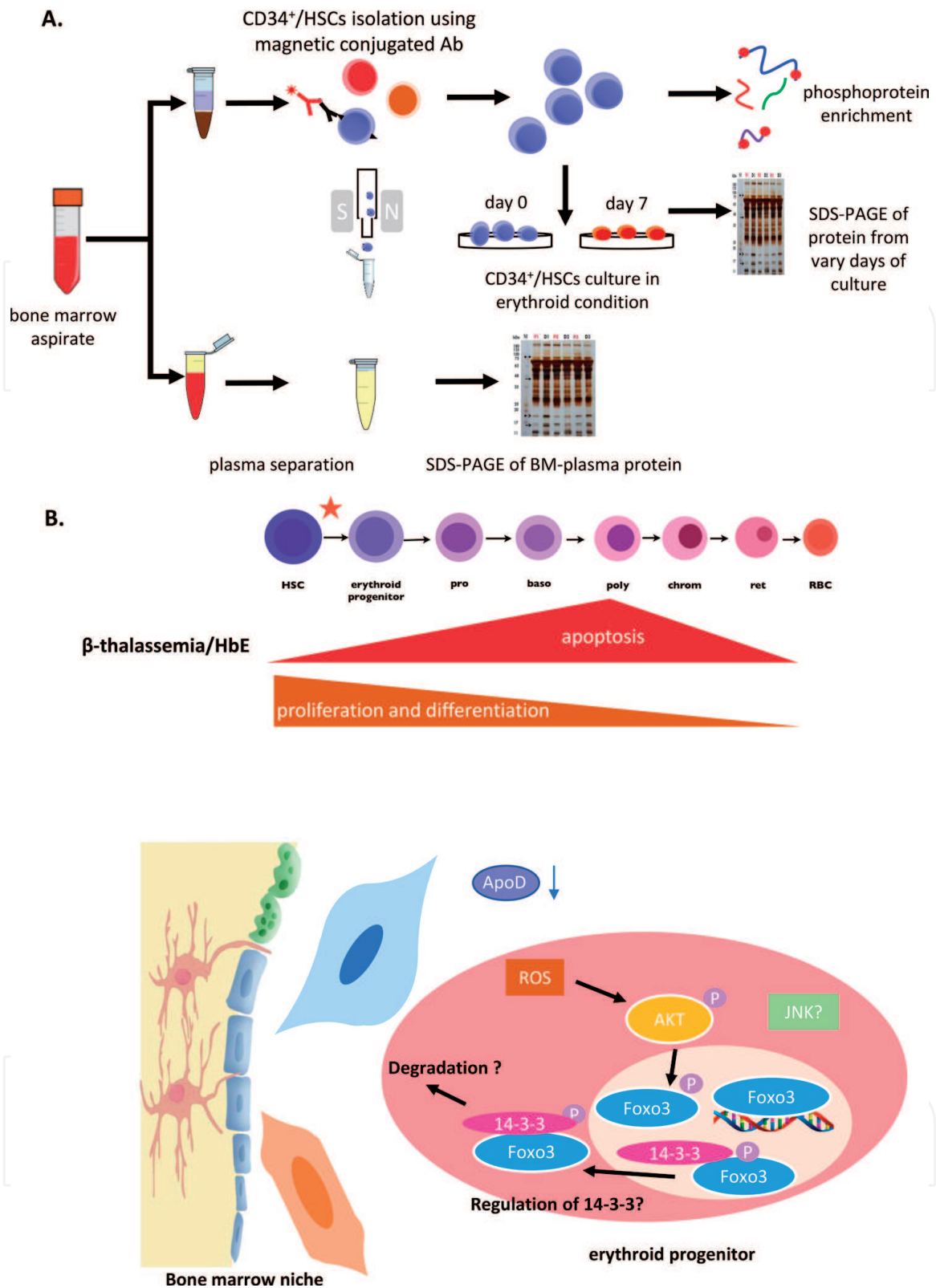


Figure 1. Diagram showed the sample preparation from bone marrow-derived CD34⁺/HSCs and bone marrow plasma prior to MS-based proteomic approaches. The three sources of proteomes for investigating ineffective erythropoiesis include CD34⁺/HSCs' phosphoproteome, dynamic differential proteome during erythroid development, and bone marrow plasma proteome (A). Schematic diagram of identified proteins and proposed signaling pathway for ineffective erythropoiesis in β -thalassemia/HbE (B).

5.3 Heat shock protein

Recent studies have suggested that the molecular chaperone or the heat shock protein maintains cellular homeostasis during erythropoiesis. Regulated by the GATA-1, the master transcriptional machinery of erythropoiesis, the heat shock

protein 70 (HSP70) plays an important role in protecting from caspase-3-mediated GATA-1 degradation [63]. Conversely, in β -thalassemia major, the alleviated free alpha globin exacerbates the oxidative damage inside erythroblast which induces the translocation of heat shock protein 70 from the nucleus to cytoplasm, leaving GATA1 unprotected [63]. The expression of heat shock protein 70 in patient group was dramatically significantly increased from day 0 to day 7, suggesting it as a contributing factor of ineffective erythropoiesis (unpublished data). Furthermore, the heat shock protein 90 that has a similar expression pattern as heat shock protein 70 and has been identified over expression from platelet-free plasma-derived microparticles proteome in β -thalassemia/HbE patients [50].

5.4 Apoptotic pathway

Erythroid progenitors undergoing terminal erythroid differentiation to generate sufficient erythroblasts and reticulocytes are impaired during ineffective erythropoiesis of β -thalassemia involving the acceleration of apoptotic cell death in erythroid precursors. The apoptotic proteins of intrinsic and extrinsic pathways have been firstly identified from the phosphoproteomic analysis of β -thalassemia CD34⁺/HSCs. This might explain why even freshly isolated HSCs of β -thalassemia bone marrow showed less survival compared to HSCs from normal donors. Phosphoproteome analysis of β -thalassemia HSCs identified an increased expression of cytochrome C (CytC), apoptosis-inducing factor (AIF), and caspase 6 (CASP6) [15]. Moreover, extrinsic apoptotic pathway plays important roles during erythroid differentiation rather than the progenitor or HSCs' stages. The analysis of thalassemia HSCs' phosphoproteome during *in vitro* differentiation to erythroid lineage of CD34⁺/HSCs revealed very high expression of death receptor proteins, such as TNF receptor-associated factor 2 (TRAF2), at day 7 but was undetectable at day 0 (unpublished data), as well as the downregulations of tumor necrosis factor ligand superfamily member 6 (FASL) and tumor necrosis factor receptor superfamily member 12A [15]. Thus, we propose that the imbalance of death signaling pathway could have a predominant effect during erythroid proliferation and differentiation in β -thalassemia. However, few studies explained how ROS and oxidative stress are related to an ineffective erythropoiesis. It remains to investigate whether ROS signaling could provoke apoptotic pathway of ineffective erythropoiesis.

5.5 The AKT/FOXO3/14-3-3 axis

After the finding of the imbalance cell death protein in CD34⁺/HSCs' proteomes from both fresh isolated and erythroid differentiated condition *in vitro*. The 14-3-3 protein was found upregulated in HSCs of β -thalassemia/HbE patients. This identified a molecular linkage between both survival and death signaling pathways [15]. An elevated ROS level in β -thalassemia erythroid cells plays an important role in the activation of AKT, potentially resulting in the repression of FOXO3 activity and reducing the cellular responses to oxidative stress. Nevertheless, AKT-mediated FOXO3 phosphorylation at Ser253 could not maintain the activation of FOXO3 activity to induce downstream gene expression of stress responses in β -thalassemia/HbE [14]. This leads to a premature apoptosis of erythroid cells. In this circumstance, activation of AKT and the translocation of FOXO3 from the nucleus to cytoplasm involve the activation of 14-3-3 which binds to FOXO3 and induces FOXO3 degradation by ubiquitination/proteasome pathway [64]. In addition to the signaling crosstalk of AKT and 14-3-3 modulated FOXO3 function, c-Jun N-terminal kinase (JNK) pathway and other posttranslational modifications, especially acetylation, promote FOXO3 transcriptional activity inside the nucleus [64, 65]. However,

the regulation of 14-3-3 among AKT and JNK signaling-mediated FOXO3 function through involving imbalance of death and survival signaling during ineffective erythropoiesis remains to be investigated (**Figure 1B**).

6. Conclusions

Proteomic analysis by MS has become an efficient tool for investigating pathophysiology of β -thalassemia/HbE overcoming limiting factors of stem cell samples, especially hematopoietic stem cells (HSCs). Integrated proteome profiling using shotgun-based and gel-based proteomic analyses of clinical bone marrow or peripheral blood samples shed light on the molecular mechanisms on ineffective erythropoiesis in β -thalassemia. Better understanding of these molecular mechanisms will help the development of novel treatment of the disease.

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Conflict of interest

The authors have no potential conflict of interest to disclose.

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