The Free Alpha-Hemoglobin: A Promising Biomarker for β-Thalassemia

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Abstract

The purpose is to review the current knowledge and methods of determination of the free alpha hemoglobin with emphasis on its potential implications in patients with beta-thalassemia (β-thal).The severity of β-thal correlates with the extent of imbalance between α- and non-α-globin chains and the amount of the free alpha hemoglobin (free α-Hb) pool in the erythrocytes. To date, there is a lack of biomarkers that convey diagnostic and prognostic values in β-thal. Our ongoing research is aimed to evaluate the potential prognostic and diagnostic implications of free α-Hb to improve the care of patients with β-thal. Our ongoing research is aimed to quantify the free α-Hb for its potential prognostic and diagnostic implications towards improving the care of patients with various β-thal types in the years to come.

Brief History

In 1963, the Greek haematologist Phaed on Fessas made observation that there were large, ragged inclusion bodies in the red cell precursors of patients with beta-thalassemia (β-thal). They were predominantly present in the peripheral blood of patients with splenectomy. He suggested that these might be α-chain and reasoned that there is excess production of α-chain that might precipitate and produce these bodies. It takes 40 years from the first description of thalassemia before these inclusion bodies were identified [1]. Among other names (free alpha hemoglobin, hemoglobin subunit alpha, alpha-globin, alpha-1 globin, alpha-2 globin, free alpha hemoglobin, free alpha globin chain, free alpha haemoglobin chain, hemoglobin alpha-1 chain and hemoglobin alpha 1 globin), free alpha haemoglobin is the preferred name. In the bone marrow, the α-globin precipitation can be seen in the earliest precursors and all through the erythroid maturation pathway [2]. On incubation of the marrow with methyl violet, it is always possible to demonstrate these inclusion bodies in the erythroblast [3]. β-thal is a consequence of the accumulation of free α-chain inclusions in the erythroid precursors that can be demonstrated in the bone marrow by both light and electron microscopy as well as in the peripheral red blood cells particularly, following splenectomy [4-7].

Methodology

A structured approach to determine the source of materials for this report was used. Key words used for websites searching were "Free alpha hemoglobin, biomarker, beta-thalassemia, alpha globin gene, alpha globin chain". The peer-reviewed literature was the principal source of information and data via ProQuest Hospital Collection and NCBI search. All searches were limited to research published in English. The Google Scholar search engine was also used in the search. The resulting literature involved over 1,800 peer reviewed journal articles. Out of the total 114 reviewed and analysed articles, 65 selected articles were included in this report. In addition, classic research and review articles were included in the references because they provided contextual material for the review.

Introduction

β-Thal is a major health problem with its impact on the nationwide human resources and burden on the families, social and the nation's economy [8]. In β-thal, the β-globin chains output range from reduced (β+) to the complete absence (β0) [9-10]. The major problem in β-thal is accumulation of excess free alpha hemoglobin (α-Hb) (previously called free/unbounded/unpaired/unmatched alpha globin chains) in the bone marrow erythroblast and peripheral erythrocytes; thus, patients with β0 thalassemia are clinically worse than patients with β+ thalassemia, and there is a general trend in this direction. In severe untreated β-thal, more than 95% of erythropoiesis is ineffective [11,12]. Concerning the globin synthesis in β-thalassemia, a small but reproducibly detectable, excess of α-globin mRNA is present in normal erythroblasts. However, β-globin mRNA is translated more efficiently than α-globin mRNA. These counterbalancing forces result in approximately equal synthesis of α- and β-globin polypeptide chains. In normal reticulocytes, α-and β-globin chains production is synchronous, though there is a very small excess of alpha chain synthesis, so that the ratio of production of alpha to non-alpha chains is 1.02 ± 0.07 [13]. Oxidation of α- or β-globin chains leads to the formation of α- or β-hemichromes respectively, and their rate of

formation determines the rate of hemolysis [3]. Because α-chains dissociate into monomers more readily than β-chains, they form hemichromes at a faster rate, which explains why β-thal is clinically more severe than α-thalassemia. Hemichromes bind to or modify various components of the mature red-cell membrane, such as band 3, protein 4.1, ankyrin and spectrin. After precipitation of hemichromes, heme disintegrates, and toxic non-transferrin-bound iron species are released. The resulting free iron catalyzes the formation of reactive oxygen species. Iron-dependent oxidation of membrane proteins with the formation of senescence antigens such as phosphatidylserine renders thalassemic red cells to become rigid, deformed and form aggregates leading to their premature removal from the circulation [14]. The precipitated globin might interfere with red cell membrane structure and function leading to leakiness of the red cells. The mechanical effects of inclusions on red cells in the circulation and spleen, explains the hemolysis in the β-thal. The α-globin chain dependent production of H_2O_2 exacerbate the loss of cellular deformability and results in impaired extrusion of reticulocytes from the marrow and shorten the survival of peripheral blood erythrocytes [15]. The severity of β-thal correlates mainly with the level of imbalance between α- and non-α-globin chains and the amount of the free α-Hb pool in the erythrocytes rather than the underproduction of Hb. In β-thal trait, there is a two-fold increase in the synthesis of alpha globin and the ratio of alpha to non-alpha globin in person with beta thalassemia intermedia is three to four folds with marked chain imbalance in β-thal major [16]. The erythroid cells ability to detoxify and remove the damaging α-Hb inclusions protein via quality control pathway are exceeded in red cells of the person with severe forms of β-thal [3]. Hemichrome-mediated oxidation of band 3 cytoplasmic domain appears to play a permissive effect for its clustering and neo-antigen exposure via dimer and tetramer formation of free α-Hb [4,13,14,17].

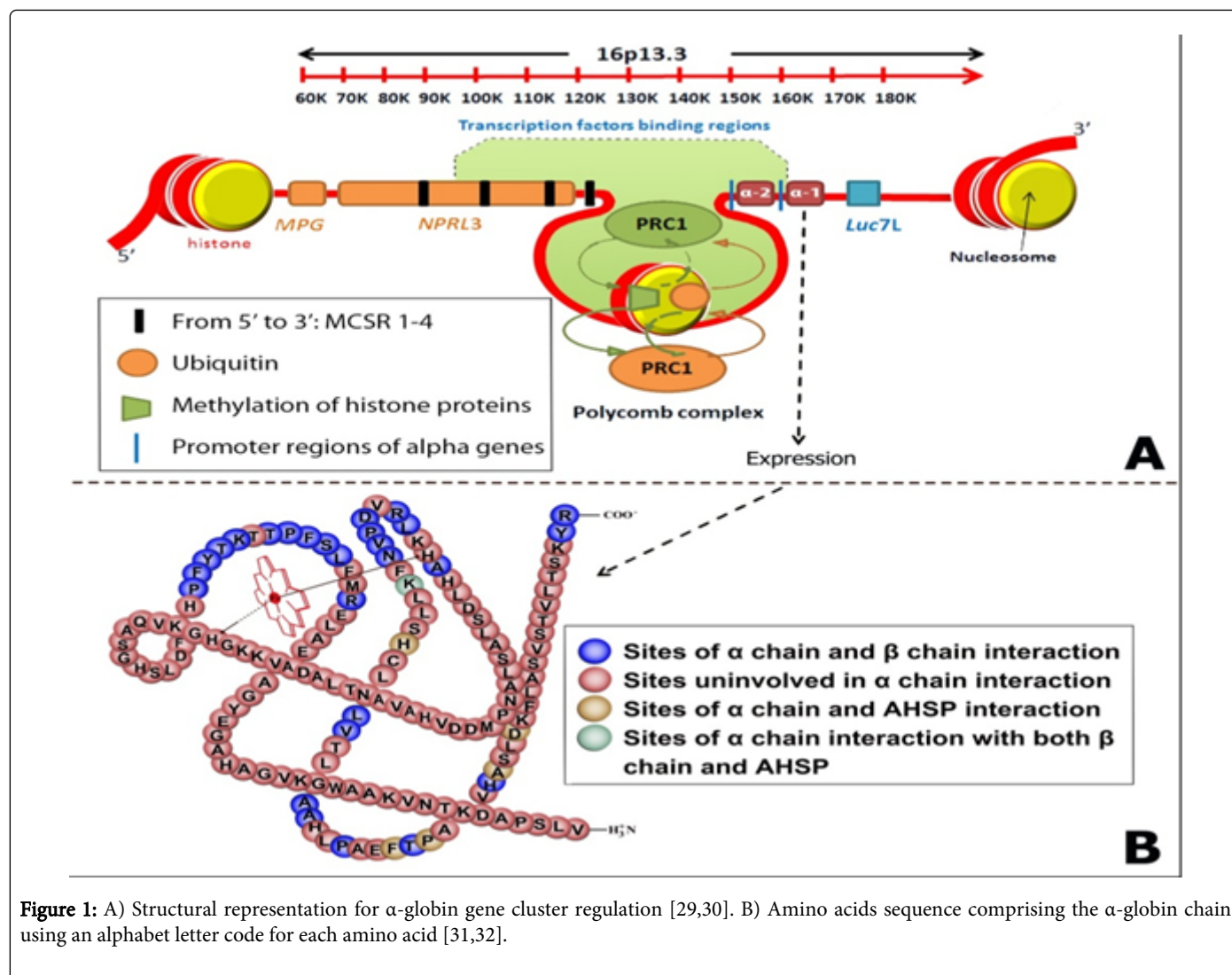## Regulation of the α-globin Gene Cluster

The human α-globin gene cluster located on chromosome 16p13.3 spans about 30 kb and includes seven loci: 5'- zeta - pseudozeta - mu - pseudoalpha-1 - α-2 - α-1 - theta - 3' flanked by highly expressed genes (*MPG, NPRL3*, and *Luc7L*) [18-20]. These genes differ slightly over the 5' untranslated regions and the introns, but they differ significantly over the 3' untranslated regions. A total of four functional and identical α-globin genes of 4 kb size exist in every cell (termed α-1 and α-2). Each of the four α-genes produces about one-quarter of the α-globin chains needed for hemoglobin synthesis. The mechanism of this coordination is unknown [21]. In normal healthy person, the number of α-genes per chromosome can range from 0 to 4 based on unequal crossing over between misaligned α-gene clusters with other recombinant events. However, the total numbers of α-genes in individuals vary in copy number from 0 to 8. [22]. In addition to the promoter region at 5' to each α-globin gene; a powerful enhancer region called the locus control region (LCR) is required for optimal gene expression. The LCR is many kilobases (so distant from the genes) upstream of the α-globin locus. The mechanism by which the promoters and LCR control α-globin gene expression is unknown. The transiently expressed zeta gene, early in development, as a component of embryonic hemoglobin is also in the α-globin locus. Both α-1 and α-2 genes encodes are 100% similar with a 100% identical 141 amino acid residues, but it has been reported that the α-gene 2 encodes twice the α-globin chains produced relative to α-1 gene which could be because of the difference in regulatory regions [23].

The α-gene transcripts and their isoforms are still being discovered to elucidate the mechanism of the globin gene interactions. The confirmed two isoforms for HBA2 and HBA1 genes have differential expression levels in the healthy individual, thus the α2- and α1-globin transcripts are present in both a long and a short isoform, initiating at positions –66bp and –37bp, respectively within the healthy population. The short isoform (–37bp) is expressed thousands times more than the longer isoform for both α2 and α1 genes. Therefore, despite the contribution of the long isoforms to the total α-globin RNA pool, the short isoforms are the principal physiological transcripts. Although the sequence differences are located at the promoter region upstream of the ATG codon, it is possible that the promoter region has functional importance and may explain the unexpected protein levels following gene alterations [24]. However, the core promoter region of the alpha gene clusters is unknown. Upstream of the α-globin genes, at about 25 to 65 kb, the presence of four highly conserved non-coding sequences referred to as multispecies conserved sequences regions (MCS–R) was reported. This MCS-R is involved in regulatory expression of α-globin genes. Three of these regions (MCS-R 1 to 3) are located within the intronic regions of adjacent NPRL3 gene [25]. Only MCS-R 2, also called HS-40 is critically involved in the expression of α-globin [18]. During the differentiation of the erythroid lineage from the pluripotent stem cells, the binding of vital transcription factors (such as, GATA1, GATA2, SCL, Sp/XKLF, NFE2) and particular cofactors (such as, FOG, pCAF, p300) to the upstream MCS-R regions and the promoter regions of the α-genes is necessary for transcription initiation. These factors are involved in chromatin modification which is followed by the HS40-mediated recruitment of RNA polymerase-II to the upstream promoters and transcription basal machinery [26,27]. Subsequently, the HS-40 mediates the formation of chromatin loop which enables the upstream transcriptional area to interact with the promoters of the α-globin genes [28]. However, PRC1 is linked to a small molecule of ubiquitin at particular site on nucleosome. This ubiquitin is an initiator for binding of PRC2 which mediates methylation at specific position in histone proteins (Figure 1) [29,30]. Though the alpha globin genes occupied a large transcriptionally active chromosomal region, they are expressed in a tissue specific manner. This tissue-specific expression is characteristic of the silent α-globin genes in non-erythroid cells by marking of particular signature (H3K27me3) [31], imposed by histone methyltransferase (EZH2). EZH2 is an essential element of repressive Polycomb complex (PRC2). In addition, other part of PRC2 namely EZH2 and SUZ12 are marked at the α-globin genes in non-erythroid cells [32]. The PRC2 makes its way by recruiting histone deacetylases (HDACs) and a second repressive Plycomb complex (PRC1) [33]. However, during erythroid development, the polycomb complex is removed aiming for activation of α-globin genes [34].

Alpha Globin Chain and its Molecular Chaperone

The molecular weight of α-globin chain is 16,000 Daltons. A small excess of α-Hb is present in healthy subjects. The free β-Hb forms relatively stable homo-tetramer, while free α-Hb exists as a structurally unstable monomer and is prone to oxidation and precipitation, contributing to the pathophysiology of β-thal [35-38]. In β-thal, the synthesis of normal α-globin chains from the unaffected α-globin gene continues as normal, resulting in the accumulation within the erythroid precursors of excess free α-globin chain, which precipitate in the red cell precursors in the bone marrow forming inclusion bodies (cytoplasmic free α-Hb).

Figure 1: A) Structural representation for α-globin gene cluster regulation [29,30]. B) Amino acids sequence comprising the α-globin chain using an alphabet letter code for each amino acid [31,32].

Excess α-globin chain precipitate on the inner surface of red cell membrane as insoluble hemichrome (Membrane-bound free α-Hb), forming smaller inclusions also called micro-Heinz bodies. The reactive oxygen species (O^{-2} and H_2O_2], in turn, oxidize adjacent RBC membrane proteins and lipids and produce superoxide at a rate 8 times that of normal hemoglobin [16,39]. The α-globin chain-dependent productions of H2O2 exacerbates the loss of cellular deformability and results in impaired extrusion of reticulocytes from the marrow and shorten the survival of the peripheral blood erythrocytes. Furthermore, the α-chain inclusions are responsible for the intramedullary destruction of the erythroid precursors and hence the ineffective erythropoiesis that underlies all β-thal. The severity of β-thal is correlates with the level of imbalance between α and non-α globin chains and the size of the free α-Hb pool in the erythrocytes. Anemia in β-thal results from a combination of ineffective erythropoiesis, peripheral hemolysis, and an overall decrease in haemoglobin synthesis [3]. Excess free α-Hb pool can lead to loss of the hemoglobin tetramer and reduce the catalytic activity of glutathione concentration which results insignificantly enhanced sensitivity to exogenous iron-derived oxidant. Thus, iron mediated toxicity contribute to the cellular pathophysiology of thalassemic cells [40,41]. It is established that imbalanced globin-chain synthesis in red cell precursors is the primary purpose of the extensive intramedullary red cell destruction, which leads to the ineffective erythropoiesis characteristic of β-thal. It follows that the imbalanced globin-chain production should occur, to a lesser extent, in heterozygous β-thalassemia. Clinical severity of β-thal is related to the ratio of alpha to beta and gamma chains which show the level of chain disequilibrium [12,42]. In severe β-thal, there is a rapidly destroyed population of red cells and another group with longer survival. There is heterogeneity in the cellular accumulation of α-globin as early as the proerythroblast stage with some precursors showing large aggregates and others, virtually none. This heterogeneity of α-globin accumulation is consistent with the extreme variation in red cells morphology and levels of cellular dehydration of thalassemic erythrocytes. Some precursors are severely affected that they die in the marrow (Apoptosis) whereas others manage to enter the peripheral blood where their survival is very heterogeneous (Figure 2). The shorter lived population has low Hb-F and contain predominantly small amount of Hb-A and α-chain precipitate. The variability in the switch on of gamma globin chains, thereby forming Hb-F and reducing the number of excess α-globin could be a reason. A modifying effect of α-chain synthesis on the synthesis of β-chains was reported.
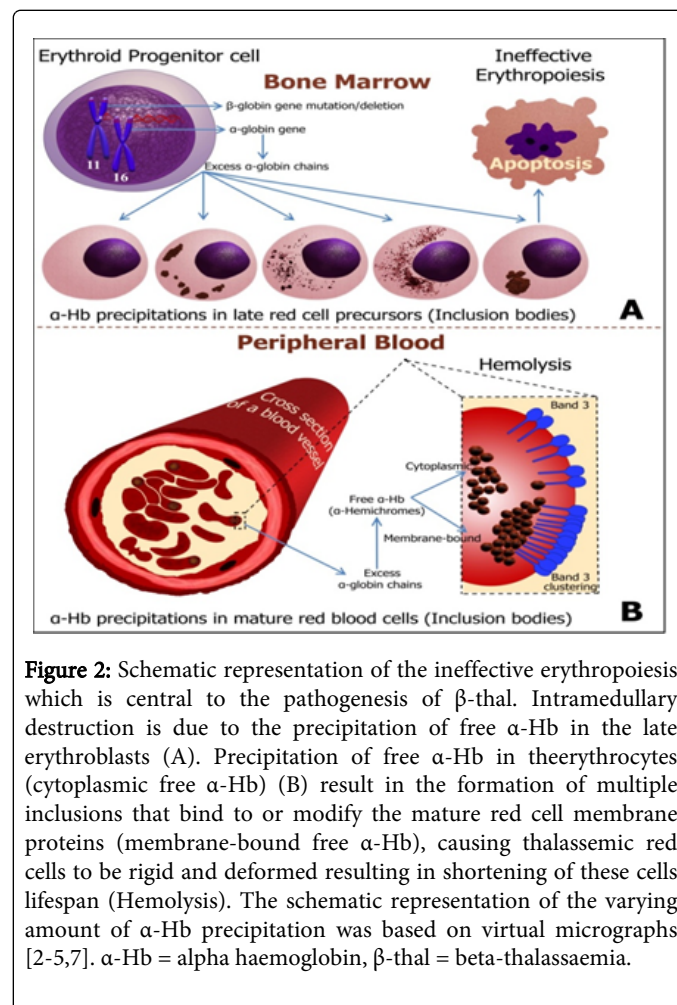
Molecular chaperones are involved in the acquisition and maintenance of the native conformation of their target proteins and thereby help prevent aggregation of these proteins. Alpha hemoglobin stabilizing protein (AHSP), the chaperone that stabilizes free α-Hb, specifically interacts with and stabilizes the free α-Hb in the absence of available β-subunit. When available, β-Hb binds more avidly to the α-subunit, displacing AHSP and forming Hb-A tetramer [12]. Unlike the β-hemoglobin (β-Hb) chains, which are soluble and form homologous tetramers, the free a-chains are highly unstable and act as active oxidants, causing apoptosis and inefficient erythropoiesis. AHSP is an abundant, erythroid-specific protein that protects free Hb from precipitation both in solution and live cells [43-45]. AHSP, a protein of 102 amino acids, is present at a high level (0.1 mM) in human red blood cell precursors. AHSP prevents the free a-Hb from forming inclusion bodies, which damage membrane structures and trigger cell apoptosis [46]. AHSP minimizes the chances for α-Hb to form cytotoxic precipitates in cells. Free α-Hb binding with AHSP gives more time for the reduced α-Hb, to be complexed by the next available β-subunit, or for the oxidized α-Hb to be reduced. Moreover, by facilitating the oxidation of the heme group and by sequestering the oxidized heme in a biochemically inert state, AHSP ensures that α-Hb does not causes oxidative damage to the red cells [12]. The interface of the α-Hb-AHSP complex involves four α-helices, two from each protein. The α-Hb interact with the β-Hb to develop a α1-β1 complex, however, the overall interactions between α-Hb and AHSP are suboptimal compared to those of the α1-β1 complex. These non-optimal α-Hb-AHSP interaction are disrupted by incoming β-Hb for the formation of functional Hb-A. In addition, the amino acid residues that participate in the α-Hb and AHSP interactions are highly conserved. These residues are selectively preserved during development, and indicating their functional significance. The role of endogenous AHSP is not limiting for α-globin detoxification in a murine model of β-thalassemia.

The mechanism by which a specialized cell coordinates high-level production of a multi-sub unit protein and protects against various synthetic imbalances was reported [47]. AHSP specifically binds to the unstable monomeric α-globin chains and prevents its precipitation when β-globin chains are unavailable. This indicates the presence of excess alpha over beta globin chains in normal erythrocytes [43]. In addition; AHSP stabilizes the nascent α-globin for HbA assembly [48]. Notably, haptoglobin preferentially binds β but not α Subunits [49].

Phenotypic Diversity and Severity of β-Thal

The three broad clinical phenotypes in patients with β-thalassaemia are major, intermedia, and minor. In addition, thalassaemia minima are the clinical phenotypes in which the carriers of β-thal gene mutation have no apparent clinical or haematological abnormalities. For more than 25 years researchers have questioned why patients who are homozygous for identical molecular defects have remarkably different phenotypes, so that, some patients need regular blood transfusion, whereas others are transfusion independent [50]. Phenotypic variability is directly affected by the level of globin chain imbalance. Patient with β-thal who also inherits one or more type of α-thalassemia (α-thal) tends to have a milder phenotype because of the reduction in the excess of α-globin chains caused by the coexistent α-thal allele. Likewise, patients with severe form of thalassemia who inherit more α-genes than normal because their parents have triplicate or quadruplicated α-gene arrangement tend to have more severe phenotypes [50]. Others with severe β-thal allele, run milder course

because of a genetically determined ability to produce more γ-globin chains, and thus Hb-F, result in a reduced level of globin chain imbalance [51,52]. The phenotypic diversity of thalassemia depends on the level of imbalance between α- and non-α-globin chains. Prompt and accurate diagnosis of non-transfusion-dependent thalassemia is essential to ensure early intervention [53]. However, thalassaemia carrier screening programmes structured among different populations varies in many aspects, including whether the programmes are mandatory or voluntary, the education and counselling provided and whether screening is offered pre-pregnancy or antenatally [54].

Figure 2: Schematic representation of the ineffective erythropoiesis which is central to the pathogenesis of β-thal. Intramedullary destruction is due to the precipitation of free α-Hb in the late erythroblasts (A). Precipitation of free α-Hb in theerythrocytes (cytoplasmic free α-Hb) (B) result in the formation of multiple inclusions that bind to or modify the mature red cell membrane proteins (membrane-bound free α-Hb), causing thalassemic red cells to be rigid and deformed resulting in shortening of these cells lifespan (Hemolysis). The schematic representation of the varying amount of α-Hb precipitation was based on virtual micrographs [2-5,7]. α-Hb = alpha haemoglobin, β-thal = beta-thalassaemia.

Free Alpha-Hemoglobin Determination

A rapid and sensitive high-performance liquid chromatography (HPLC) method for the detection of human globin chains in the blood was described [55,56]. There is a well-known possibility to incorporate radioactive amino acid such as 3H-leucine into hemoglobin in red cells, incubated in vitro, provided there are a sufficient number of reticulocytes from rabbit's blood [53]. Experiment in human is hindered by the low reticulocyte count in the blood of thalassemia patients and the need for handling of radionuclides and chromatographic separation of globin chains before quantification by liquid scintillation counting. Human reticulocytes are labeled *in-vitro* and then lysed. Thereafter, the whole lysate converted to globin. It is possible to measure the total amount of α and β chain synthesized, with the recovery of radioactivity and protein in excess of 95% [51].

The α/β-globin mRNA ratio in normal subjects is 1.2 to 2.3 fold increase of α-globin mRNA over β-globin mRNA. The highest α/β-globin mRNA ratios were seen in β^0- thal/Hb E syndrome and the ratios correlated with the severity of the anemia [57]. The combination of Hb-E with β-thal profoundly affected α/β-globin mRNA ratio and anemia severity. Subject with a lower α/β-globin mRNA ratio had a less severe anemia than one with a higher ratio [58,59]. A successful application of recombinant AHSP to measure the free α-Hb pool in hemolysate of patients with β-thal and normal individuals was reported, and also demonstrates that the free α-globin pool in β-thal patients depends not only on the β-globin genotype but also on α-globin genotype [60]. To date, free α–Hb measurement has not been established for diagnosing or prognosis determination of patients with β-thalassaemia.

Recommendation

At 2002, US researchers reported that stabilizing protein could alleviate blood disorder primarily in thalassaemia [61]. The quantifiable free α–Hb pool is correlated with the severity of the disease; thus, free α–Hb pool is a promising biomarker of diagnostic relevance as well as prognostic value in β-thal syndrome. Development of cost effective and point of care screening of β-thal in diverse population contributes towards decreasing the cost and workup for screening and confirming the diagnosis of β-thal phenotypes in a world-wide base. Free α–Hb pool quantification may be applicable on the splenic biopsy for assessing the severity of diseases before considering splenectomy for patients with β-thal major. Quantification of free α–Hb in the cord blood sample offers early diagnosis of β-thal in new born. In the current limited tools for monitoring the efficiency of various treatment modalities of β-thal, bone marrow aspirate is another material for further research projects. Furthermore, enhancement of the classification system of the severity of β-thal is possible by the inclusion of the free α–Hb pool quantification in the scoring system [6].

Our ongoing research is aimed to quantify the free α-Hb for its potential prognostic and diagnostic implications towards improving the care of patients with various β-thal phenotypes in the years to come. Further study toward detection and quantification of the free alpha globin chain using the microfluidic-based electrophoresis system is recommended. Thalassaemias originate mostly in low-income countries where infants would not have survived to be diagnosed or treated. Moreover, with increasing migration and travel, a genetic disease that was rare in northern Europe, Australia, and North America is now becoming more common, and systems for genetic counselling, prenatal diagnosis, and life-long medical care are needed [49]. A cost efficient test with diagnostic and prognostic clinical utility provides early diagnosis and improves the treatment-option determination at initial stage of the disease may contribute towards decreasing the burden of patients with β-thal on the limited human blood resources and blood banking budget. Development of cost effective immunodiagnostic kit for quantification of the free α–Hb offers an alternative, for the clinician to use in the future, to make clinical decisions without the current diagnostic and prognostic limitations in the management of β-thal. Measurement of the free α-Hb in the blood may prove helpful for the initial distinction of different types of β-thal as well as in identifying and confirming the diagnosis of the severe form of the disease.

The most important development for the next few years might be the development of cheap accurate prenatal testing, which has been effective when applied systematically [62,63]. The free α-Hb assay may provide a more precise scale of severity within a given genotype, serve to refine the diagnosis for heterozygous forms, provides better classification of intermediate β-thal, could also be used in cases of atypically β-thal with a normal percent of Hb A2, which are phenotypically indistinguishable from other causes of hypochromia and microcytosis, facilitate an efficient and cost-effective screening program for hemoglobinopathies in diverse populations, applied to all diseases with an imbalance of globin synthesis, and moreover, will allow monitoring of the development of this imbalance in response to various emerging therapeutic modalities [60,64-66].

Acknowledgment

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