DETERMINATION OF ALPHA HEMOGLOBIN STABILIZING PROTEIN (*AHSP)* GENE EXPRESSION AND OXIDATIVE STRESS PARAMETERS IN HbE/BETA-THALASSEMIA

NUR SURAYA BINTI CHE YAACOB

UNIVERSITI SAINS MALAYSIA

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by

NUR SURAYA BINTI CHE YAACOB

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LIST OF SYMBOLS

%	Percentage
/	Or
~	Approximately
<	Less than
>	More than
±	Plus minus
π	Pi
Δ	Increment
^{o}C	Celcius
bp	Base pair
CD	Codon
OD	Optical density
fL	Femtolitre
g	Gram
g/dL	Gram per decilitre
L	Litre
mins	Minutes
mL	Millilitre
n	Nano
ng	Nanogram
ng/µL	Nanogram per microlitre
ng/mL	Nanogram per milliliter
nm	Nanometer
mg/kg	Milligram per kilogram

pg	Picogram
rpm	Revolution per minute
RT	Room temperature
sec	Second
V	Voltage
α	Alpha
β	Beta
β^{+}	Beta plus (reduction of β-globin chain)
β^{++}	Beta silent
β^{0}	Beta node (complete absence β-globin chain)
γ	Gamma
δ	Delta
µ	Micro
µL	Microlitre
µM	Micromolar

LIST OF ABBREVIATIONS

AHSP	Alpha Haemoglobin Stabilizing Protein
ActR	Activin receptor
ASC	Ascorbic acid
ATF4	Activate transcription factor 4
CAT	Catalase
cDNA	complementary DNA
CHA	Chronic hemolytic anaemia
CRL	Central Research Laboratory
CuZn	Zinc-copper
DNA	Deoxyribonucleic acid
EDRF	Erythroid differentiation-related factor
EDTA	Ethylenediaminetetraacetic acid
eIF2	Eukaryotic initiation factor 2
ELISA	Enzyme-linked immunosorbent assay
Fe (II)	Iron (II)
Foxo 3	Forkhead box O3
GI	Gastrointestinal
GR	Glutathione reductase
GSH	Thiol glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HbA	Adult haemoglobin
HbE	Haemoglobin E
HbF	Fetal haemoglobin
HbH	Haemoglobin H
HIP	Hypoxia-inducible factor
HO-1	Heme oxygenase-1
HPLC	High-performance liquid chromatography
HRPZ (II)	Hospital Raja Perempuan Zainab (II)
Jak	Janus kinase

KPP	Klinik Pakar Perubatan
LAP	Lysosome-autophagy pathways
LIP	Labile iron pool
MDA	Malondialdehyde
metHb	methaemoglobin
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO·	Nitric oxide radical
NTBI	Non-transferrin-bound iron
NTDT	Non transfusion dependent
$\cdot O_2$	Superoxide radical
$O^{\cdot -2}$	Ion radical
O_2	oxygen
O_2^-	Superoxide ion
OH·	Hydroxyl radical
oxyHb	oxyhaemoglobin
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol myristic acetate
PMNs	Polymorphonuclear neutrophils
PMRS	Plasma membrane redox in system
PRDX2	Peroxiredoxin-2
PUFA	Polyunsaturated fatty acid
QTL	Quantitative trait loci
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

TDT	Transfusion dependent
TfR1	Surface transferrin receptor
TGF	Transforming growth factor
TI	Thalassaemia intermedia
TM	Thalassaemia major
tRNA	Transfer ribonucleic acidma
USM	Universiti Sains Malaysia

LIST OF APPENDICES

PENENTUAN EKSPRESI GEN ALPHA HEMOGLOBIN STABILIZING PROTEIN (*AHSP*) DAN PARAMETER TEKANAN OKSIDATIF DALAM HbE/BETA-TALASEMIA

ABSTRAK

Beta-Talasemia adalah gangguan sintesis genetik hemoglobin yang dicirikan oleh sintesis rantai β-globin yang berkurang atau tidak ada sehingga menyebabkan ketidakseimbangan tetramer. Alpha Hemoglobin Stabilizing Protein (AHSP) bertindak sebagai pendamping molekular untuk α-globin dan menstabilkan α-globin bebas untuk menghalagngnya daripada mendakan dan membentuk produk sampingan iaitu spesies oksigen reaktif dengan kerosakan oksidatif dan seterusnya membentuk mendapan intraselular sehingga menyebabkan tekanan oksidatif. Ujian PCR dan ELISA digunakan untuk menentukan ekspresi AHSP dan tekanan oksidatif (aktiviti SOD dan kepekatan MDA) dalam sampel darah. Ekspresi AHSP signifikan dalam parameter keparahan penyakit (p=0.001), kebergantungan transfusi (p=0.033), profil molekul (p=0.035) dan usia (p=0.034), aktiviti SOD signifikan dalam keparahan penyakit (p=0.005), kebergantungan transfusi (p=0.001), tahap ferritin serum (p=0.005) dan usia (p=0.040), dan kepekatan MDA signifikan dalam keparahan penyakit (p=0.003), kebergantungan transfusi (p=0.001), status splenektomi (p=0.002), tahap feritin serum (p=0.002) dan usia (p=0.015). Kami mendapati bahawa ekspresi AHSP berkorelasi secara signifikan dengan HbF (p=0.033) dan kekerapan pemindahan darah setiap tahun (p=0.011) sementara aktiviti SOD berkorelasi dengan usia (p=0.033), HbF (p=0.009) dan kekerapan pemindahan darah setiap tahun (p=0.004). Manakala, kepekatan MDA berkorelasi dengan usia (p=0.008) dan ferritin serum (p=0.022). Di samping itu, kajian korelasi dinilai antara ekspresi AHSP dengan aktiviti SOD dan kepekatan MDA di mana ekspresi AHSP berkorelasi secara

signifikan dengan kedua parameter apabila masing-masing menunjukkan nilai p=0.002 dan p=0.001. Oleh itu, kami menyimpulkan bahawa AHSP dapat menjadi mekanisme kompensasi sekunder dalam sel darah merah untuk mengimbangi lebihan rantai α-globin sehingga dapat mengurangkan tekanan oksidatif pada individu HbE/Beta-Talasemia. Pengubah AHSP dan parameter tekanan oksidatif dapat memberikan gambaran masa depan mengenai peranannya dalam patogenesis penyakit.

DETERMINATION OF ALPHA HEMOGLOBIN STABILIZING PROTEIN (*AHSP*) GENE EXPRESSION AND OXIDATIVE STRESS PARAMETERS IN HbE/BETA-THALASSEMIA

ABSTRACT

Beta-Thalassaemia is the genetic disorders of haemoglobin synthesis characterized by reduced or absent β-globin chain synthesis thus lead to imbalance of tetramer. The Alpha Haemoglobin Stabilizing Protein (AHSP) acts as a molecular chaperone for α-globin by stabilizing free α-globin preventing it from precipitating and forming reactive oxygen species byproducts with subsequent oxidative damage and the formation of intracellular precipitates thus led to oxidative stress. Real time PCR and ELISA assay were used to determine the *AHSP* expression and oxidative stress parameters (SOD activity and MDA concentration) in blood sample respectively. Expression of *AHSP* significant in disease severity (p=0.001), transfusion dependency (p=0.033), molecular profile (p=0.035) and age (p=0.034), SOD activity significant in disease severity (p=0.005), transfusion dependency (p=0.001), serum ferritin level (p=0.005) and age (p=0.040), and MDA concentration significant in disease severity (p=0.003), transfusion dependency (p=0.001), splenectomy status (p=0.002), serum ferritin level (p=0.002) and age (p=0.015). We found that *AHSP* expression was significantly correlated to HbF (p=0.033) and frequency of blood transfusion per year (0.011) while SOD activity significantly correlated with age (p=0.033), HbF (p=0.009) and frequency of blood transfusion per year (p=0.004). On the other hand, MDA concentration was significantly correlated with age (p=0.008) and serum ferritin (p=0.022). In addition, correlation study was evaluated between *AHSP* expression with SOD activity and MDA concentration in which *AHSP* expression was significantly correlated with both parameters when p=0.002 and p=0.001 respectively. Thus, we

concluded that AHSP could be a secondary compensatory mechanism in red blood cells to counterbalance the excess α-globin chains thus reduced the oxidative stress in HbE/Beta-Thalassaemia individuals. AHSP modifier and oxidative stress parameters give the future insight on its role in disease pathogenesis.

CHAPTER 1

INTRODUCTION

1.1 Background and rational of study

Thalassaemia is defined as absence or reduce of one or more globin chain of human Hb while beta-thalassaemia is the genetic disorders of haemoglobin synthesis characterized by reduced or absent β-globin chain synthesis, resulting in decrease Hb and decrease RBC production (Galanello & Origa, 2010; Nienhuis & Nathan, 2012). Thalassaemia major and thalassaemia intermedia are included as the phenotypes of homozygous or heterozygous compound of beta-thalassaemia. Individuals with thalassaemia major prefer come to medical attention within first two years of life and require regular RBC transfusions to survive while for thalassaemia intermedia, patients who present later and do not require regular transfusion. Heterozygous beta-thalassaemia results in the clinically silent carrier state. A great range of in terms of diversity of phenotypes and spectrum of severity of HbE/Beta-Thalassaemia and HbC/Beta-Thalassaemia are exhibited (Galanello & Origa, 2010).

Haemoglobin E-beta thalassaemia (HbE/Beta-Thalassaemia) resulted from interaction of HbE and beta-thalassaemia and a most common type of thalassaemia seen in Malaysia. It is known as genotype responsible for approximately one-half of all severe beta-thalassaemia and is characterized by marked clinical variability, ranging from a mild and asymptomatic anaemia to a life-threatening disorder that required transfusions from infancy. Haemoglobin E-beta thalassaemia (HbE/Beta-Thalassaemia) is known as genotype responsible for approximately one-half of all severe beta-thalassaemia and is characterized by marked clinical variability, ranging from a mild and asymptomatic anaemia to a life-threatening disorder that required transfusions from infancy (Olivieri, Pakbaz & Vichinsky, 2011a). The co-inheritance

of a beta-thalassaemia allele from one parent and the structural variant Haemoglobin E from the other is the result for HbE/Beta-Thalassaemia. Substitution in codon #26 of G → A of the beta-thalassaemia globin gene is the result of Haemoglobin E that produced structurally abnormal haemoglobin as well as activated a cryptic splice site in which resulting in abnormal messenger RNA (mRNA) processing. Many factors such as reduced β chain synthesis that results in globin chain imbalance, ineffective erythropoiesis, apoptosis, oxidative damage and shortened red blood cell survival are related to the pathophysiology of HbE/Beta-Thalassaemia (Pootrakul *et al.*, 2000).

The cellular apoptosis is led by the formation of α-globin inclusions that occurs early during erythropoiesis and peaks in the polychromatophilic erythroblasts (Mathias *et al.*, 2000). Thus, a protein complex of interaction between α-globin with its molecular chaperon which is alpha-haemoglobin stabilizing protein (AHSP) was formed before it released to interact with β-globin in forming the haemoglobin tetramer (Yu *et al.*, 2007; Weiss & Santos, 2009). The role of AHSP is facilitates folding of α-globin and prevents the formation of misfolded aggregates. Microcytosis and anaemia in humans is associated with α-globin mutations that impair interaction with AHSP (Yu *et al.*, 2009). Molecular aggregates were formed by α-globin which precipitate, forming inclusions that damage the cell membrane and the membranes of intracellular organelles once the capacity of AHSP is exceeded.

The aggregation of excess α-globin (associated with toxic heme) and formation of inclusion bodies (hemichromes) within the cell leads to formation of reactive oxygen species (ROS) thus resulting in oxidative stress and within mature red blood cells and immature developing erythroblasts. ROS attack cause free radical and lipid peroxidation and formation of an array of unwanted product such as Malondialdehyde

(MDA), a major lipid peroxidation product. Oxidative stress may aggravate symptoms of hemolytic anaemia such as beta-thalassaemia (Figure 1.1).

Thalassaemia is known as one of the health burden in Malaysia that needed for prolong treatment. Despite intensive research on molecular defect caused HbE/Beta-Thalassaemia, however limited study conducted to define the association HbE/Beta-Thalassaemia with Alpha Haemoglobin Stabilizing Protein (*AHSP*) expression and oxidative stress in beta-thalassaemia pathophysiology. Level of *AHSP* expression and the oxidative stress important act as parameters for disease severity indication in HbE/Beta-Thalassaemia patients. Understanding AHSP and its relation to oxidative stress provides a theoretical basis for new strategies to inhibit the damaging effects of free α-globin that accumulates in β-thalassaemia. The knowledge on the disease severity can be expended as to create the future insight to ameliorate disease and create potential application for targeted therapy. Hence, this study will focus on *AHSP* expression and oxidative stress level in HbE/Beta-Thalassaemia based on disease severity.

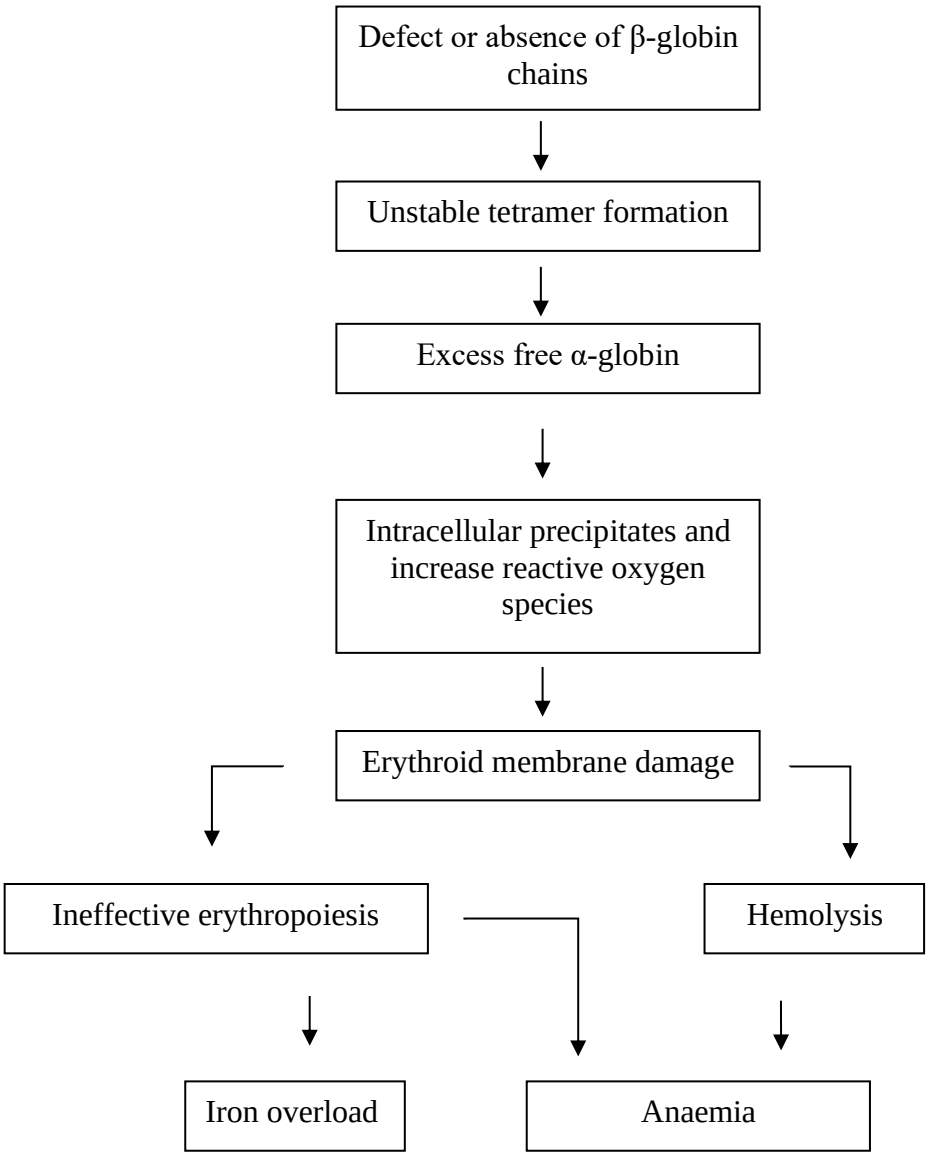

Figure 1.1 Pathophysiology of Beta-Thalassaemia

1.2 Problem statement

To date, not a single study has investigated the correlation between *AHSP* and oxidative stress parameters. The critical or main point of the research is to identify the level of *AHSP* expression and the oxidative status thus to correlate between *AHSP* and oxidative status in HBE/Beta-Thalassaemia.

1.3 Hypothesis

Level of *AHSP* expression and oxidative stress play a major role in the pathophysiologic complications of HbE/Beta-Thalassaemia patients. Correlation between *AHSP* expression and oxidative stress level in HbE/Beta-Thalassaemia patients provide a clear image on pathophysiology of thalassaemia disease.

1.4 Research questions

1. What is the demographic, clinical and laboratory data of HbE/Beta-Thalassaemia patients?
2. What is the level of AHSP expression and oxidative stress parameters in several parameters of HbE/Beta-Thalassaemia patients?
3. How AHSP expression and oxidative stress parameters correlate with several clinical parameters in HbE/Beta-Thalassaemia?
4. How AHSP expression correlate with oxidative stress parameters in HbE/Beta-Thalassaemia?

1.5 Objectives

1.5.1 General objective

To determine the Alpha Haemoglobin Stabilizing Protein (*AHSP*) expression and oxidative stress parameters level in HbE/Beta-Thalassaemia patients.

1.5.2 Specific objectives

1.5.2(a) To evaluate the demographic, clinical and laboratory data of HbE/Beta-Thalassaemia.

1.5.2(b) To determine *AHSP* expression and Oxidative Stress parameters in several clinical parameters of HbE/Beta-Thalassaemia.

1.5.2(c) To correlate the *AHSP* expression and Oxidative Stress parameters with clinical parameters HbE/Beta-Thalassaemia.

1.5.2(d) To correlate *AHSP* expression with Oxidative Stress parameters in HbE/Beta-Thalassaemia.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to haemoglobin

It has been well established in medical literature that the inhaled oxygen in lungs is transported to the rest of the body by a tetrameric protein called haemoglobin (Hb). Four molecules of oxygen form an oxidized complex with the iron in Hb which facilitates this transport. Out of all the known isomers, HbA1, comprising of 2 alpha subunits and 2 beta subunits (α2β2) forms the majority share. The abundance of α-globin in plasma means the output ratio too is considerably higher in normal human beings as compared to β-globin (Bunn, 1986; Schechter, 2008).

Excess free α-globin subunits have a high inherent instability and appear to bind together, leading to self-aggregation and precipitation. This therefore affects the normal erythropoiesis that further caused apoptosis and the nucleated red blood cells (RBCs) are let into the circulation and has adverse effects on the development of serious human diseases (Kong *et al.*, 2004a). Point mutations or minor deletions in the chromosome 11 β-globin gene (HBB) leads to Beta-Thalassaemia. It is characterized by decrease or the complete absence of β-globin subunit in the tetramer (Alaithan, Azeez & Francis Borgio, 2018). Therefore, a surcharge of unknown α-globin subunits that engage in further aggregation and precipitation worsens the scenario (Shang & Xu, 2017; Mettananda & Higgs, 2018; Mankhemthong *et al.*, 2019).

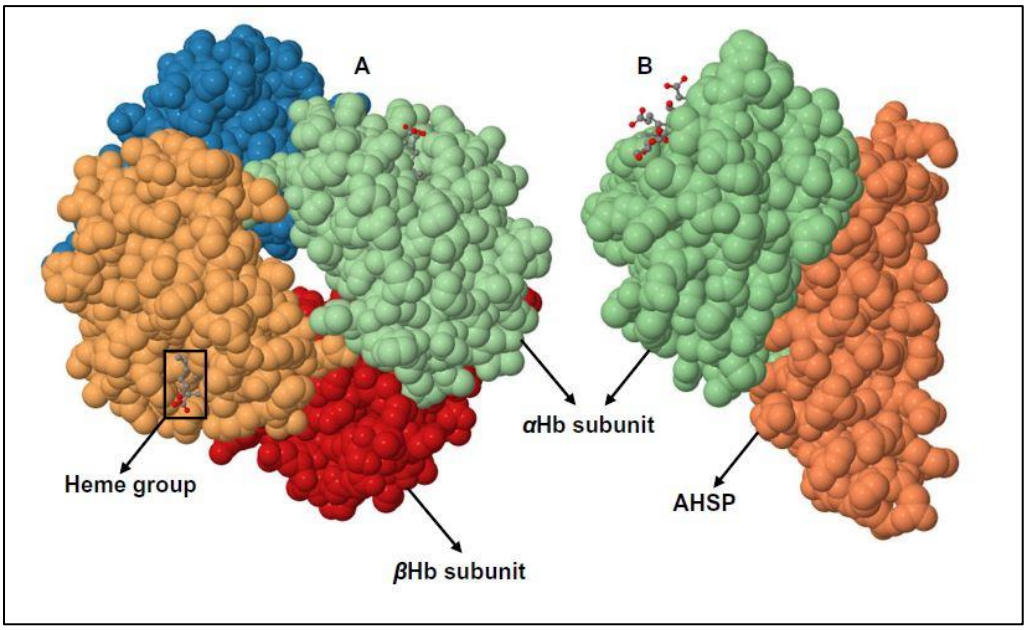

Figure 2.1 Structure of tetrameric haemoglobin molecule. Adapted from (Che Yaacob *et al.*, 2020)

2.2 Beta-thalassaemia

Beta-thalassaemia is the genetic disorders of haemoglobin synthesis characterized by reduction or absence of the synthesis machinery of β-globin synthesis, decreased RBC production and subsequently anaemia (Galanello & Origa, 2010; Nienhuis & Nathan, 2012).

Transmission of thalassaemia are by autosomal recessive traits and Beta-thalassaemia can be differentiated into different groups which are Thalassaemia major, Thalassaemia intermedia and Thalassaemia minor. The other group is beta-thalassaemia also having Hb abnormalities which are HbC/ Beta-thalassaemia, HbE/ Beta-thalassaemia and HbS/ β--thalassaemia (clinically mimics sickle-cell disease(SCD)) while the other group is a result of continuation of the expression of fetal Hb and Beta-thalassaemia (autosomal dominant forms and with miscellaneous manifestations), Beta-

thalassaemia-tricothiodystrophy and thalassaemia in association with X-linked thrombocytopenia (Galanello & Origa, 2010).

These anomalies in the increasing order of their clinical severity are classified into three groups which are beta-thalassaemia carrier state, thalassaemia intermedia and thalassaemia major. Heterozygous inheritance beta-thalassaemia which is beta-thalassaemia carrier state is clinically asymptomatic. On the other hand, thalassaemia major (TM) patients require blood transfusion for survival. Thalassaemia intermedia (TI) is a mosaic of heterogenous disorders that mimic thalassaemia and can vary in presentation from asymptomatic to something as severe as dependence on transfusion.

The potency in expression of severe features in beta-thalassaemia depends on the amount of discrepancy between the two globin chains in the molecule i.e. α and non-alpha. Normally, the latter chain also comprises of the gamma subunit which is a component highly specific of fetal Hb (alpha2-gamma2). In adults however, its quantity is sparse. These non-α-globin are present in higher amounts in beta-thalassaemia syndromes. With the absence or reduced β-globin, the unpaired α-globin get precipitated. This precipitate damages the cell membrane by oxidation and leads to cell death in the precursor stage of these RBCs (Cao & Galanello, 2010).

2.2.1 Epidemiology

Beta-thalassaemia is one of the common autosomal recessive disorders which is commonly encountered in the Mediterranean countries, Middle East, Central Asia, India, Southern China, Far East and along with the north coast of Africa and in South America. This geographical distribution is postulated to be the result of endemicity of Plasmodium falciparum malaria in these regions (Cao & Galanello, 2010).

2.2.2 Clinical features

TDT and NTDT exhibit phenotypical characteristics of homo- or heterozygous beta-thalassaemia wherein the patients with TDT genotype require hospitalization within the first couple of years of life and are transfusion dependent for the rest of their lifetime. Individuals with NTDT present relatively late to a medical institution and require transfusions less regularly than former. The carrier state is a heterozygous beta-thalassaemia. This probably outlines the reason behind the diversity of phenotypical presentation and the range of clinical severity of these conditions (Galanello & Origa, 2010).

2.2.2(a) Beta-thalassaemia major

It is characterized by the inability of the affected infants to thrive due to feeding difficulties and diarrhea, with the severe anaemia causing progressive pallor. Subsequent irritability, recurrent fever episodes due to immunocompromised state and abdominal enlargement secondary to hepatosplenomegaly are common between the age of 6 months to 2 years. Signs such as retardation of growth, muscular inadequacies, genu valgum, ulceration over lower limbs due to venous stasis, visceral swellings and skeletal malformations due to extramedullary hematopoiesis and inadequate transfusion (causing bone marrow expansion) respectively are noted. Regular transfusion maintaining the Hb levels between 9.5 to 10.5 gm% has been reported to normalize growth and development till the child reaches the age 10 to 12 years (Galanello & Origa, 2010).

One of the possible complications associated with overt transfusions in these children is iron overload. This may lead to paradoxical retardation of growth, cardiac manifestations (arrythmias or dilated cardiomyopathy), hepatic manifestations (cirrhosis and fibrotic changes), endocrine imbalance (diabetes mellitus, thyroid,

parathyroid, pituitary and adrenal insufficiency) as well as sexual immaturity (Galanello & Origa, 2010). Some of the more chronically morbid sequalae of iron overload include hypersplenism, hepatitis B, hepatitis C, HIV, Deep venous thrombosis and compromised bone mineral density. The underlying liver pathologies subject the patient to a high predisposition to hepatocellular carcinoma (Galanello & Origa, 2010).

2.2.2(b) Beta-thalassaemia intermedia

Patients affected by NTDT suffer from a transfusion independent form of anaemia which can be managed by intermittent transfusions which leads to this condition being diagnosed in these individuals at an older age than that of TDT. The range of severity is extremely huge with one end of the spectrum presenting between the ages 2 to 6 years and having retardation of growth and development while the other end of the spectrum comprises of people with absolutely no clinical features except a mild form of anaemia even till adulthood (Galanello & Origa, 2010).

The chronic anaemia in these patients leads to a compensatory bone marrow hypertrophy and extramedullary erythropoiesis which leads to skeletal deformities in the face, pathological long bone fractures due to osteoporosis and irregular masses in the spleen, liver, lymph nodes, chest and vertebral column. The splenomegaly is attributed to the role of the organ in filtering out the non-physiological RBCs aka graveyard of red blood cells (Galanello & Origa, 2010).

The erythropoietic masses in the vertebral column cause pressure symptoms by impingement onto the spinal cord leading to paraplegia. Similarly, mediastinal masses are reported to cause pressure symptoms in the chest. Gallstones are formed due to ineffective erythropoiesis and peripheral hemolysis which occurs more frequently in these patients as compared to those affected by TDT (Galanello *et al.*, 2001). Another

such feature encountered more in NTDT is the development of ulcers in the leg due to stagnation of venous blood flow secondary to thrombosis of deep veins, portal veins and their sequalae such as stroke and pulmonary embolism (Taher *et al.*, 2008).

Despite the chronic anaemia causing compensatory increase in the intestinal absorption of iron, the iron overload in these patients is not as marked as that in TDT. Hence, endocrinal, hepatic, gonadal and sexual manifestations seen in the latter are less marked in NTDT. While those never or minimally transfused are at risk of developing hemolytic alloantibodies and erythrocyte autoantibodies, the blood transfusions are necessary during pregnancy, however the risk of intrauterine growth retardation has been reported despite judicious transfusion protocol (Nassar *et al.*, 2008).

Cardiovascular manifestations do persist in NTDT although not as severe as in TDT. The high-output state owing to the chronic anaemia causes pulmonary hypertension although systolic left ventricle function is usually preserved. Degradation of the elastic lamina of the arterial wall and calcium deposition in this patients may cause a diffuse connective tissue disorder with vascular manifestation that is known as pseudoxanthoma elasticum (Aessopos, Farmakis & Loukopoulos, 2002).

2.2.2(c) Beta-thalassaemia minor

Being a recessive trait, there is low percent of each pregnancy having a homozygous combination with clinical features whereas those with a heterozygous allele form are carriers and may only be diagnosed after incidental finding of persistently mild anaemia (Galanello & Origa, 2010).

2.2.2(d) Dominant beta-thalassaemia

Inability of the marrow to produce normal β-globin chains or the predisposition to producing unstable beta variants owing to an underlying mutation leads to the

formation of an extremely unstable Hb tetramer which eventually precipitates and causes apoptosis of the erythroid precursor cells. These mutations are clinically exhibited even in the heterozygous allelic states of some individuals which is deemed as dominant beta-thalassaemia. Individuals affected by NTDT, with both parents having a normal hematological profile or belonging to families with a pattern of autosomal dominant inheritance of NTDT phenotype are generally reported to have a highly unstable Hb tetramer compound (Galanello & Origa, 2010).

2.2.2(e) Beta-thalassaemia associated with other features

In rare instances of beta-thalassaemia, the defect is not in the beta gene cluster. beta-thalassaemia trait is associated with other mutations such as a molecular lesion found either in gene encoding the transcription factor TFIIH (beta-thalassaemia trait associated with tricothiodystrophy) or in the X-linked transcription factor GATA-1 (X-linked thrombocytopenia with thalassaemia) (Freson *et al.*, 2002).

2.2.3 Etiology

The point mutations is the large majority that have been reported in translationally significant areas of the β-globin gene (Giardine *et al.*, 2007). A reduced or absence of β-globin chains is caused by the respective mutations. However, deletions of β-globin gene are uncommon. A list of common mutations based on the severity and ethnic distribution is shown in Table 2.1.

Table 2.1 Common types of beta-thalassaemia: severity and ethnic distribution (Galanello & Origa, 2010)

Population	B-gene mutation	Severity
Indian	-619 del	β^0
Mediterranean	-101 CTT	β^{++}
Black	-88 CTT	β^{++}
Mediterranean; African	-87 CTG	β^{++}
Japanese	-31 ATG	β^{++}
African	-29 ATG	β^{++}
Southeast Asian	-28 ATC	β^{++}
Mediterranean; Asian Indian	IVS1-nt1 GTA	β^0
East Asian; Asian Indian	IVS1-nt5 GTC	β^0
Mediterranean	IVS1-nt6 TTC	$\beta^{+/++}$
Mediterranean	IVS1-nt1 10 GTA	β^+
Chinese	IVS2-nt654 CTT	β^+
Mediterranean	IVS2-nt745 CTG	β^+
Mediterranean	codon 39 CTT	β^0
Mediterranean	codon 5-CT	β^0
Mediterranean; African-American	codon 6-A	β^0
Southeast Asian	codon 41/ 42 –TTCT	β^0
African-American	AATAAA to AACAAA	β^{++}
Mediterranean	AATAAA to AATGAA	β^{++}
Mediterranean	codon 27 GTT Hb (Hb Knossos)	β^{++}
Southeast Asian	codon 79 G>A (Hb E)	β^{++}
Malaysia	codon 19 G>A (Hb Malay)	

β^0: complete absence of β-globin on the affected allele
β^+: residual production of β-globin (around 10%)
β^{++}: very mild reduction in β-globin production

2.2.3(a) Genetic modifiers

Variations in the gene leading to differences in disease phenotype is the definition of modifier genes. Primary genetic modifiers influence the clinical severity of the disease including genetic variants that tend to ameliorate the globin chain imbalance leading to a milder form of thalassaemia in homozygous beta-thalassaemia. Factors of co-inheritance of α-thalassaemia, genetic determinants and the presence of silent or mild beta-thalassaemia alleles are associated with a high residual output of β-

globin that are able to sustain a continuous production of gamma globin chains (HbF) in adult life (Galanello & Origa, 2010).

The *"per se"* of the gamma globin gene output is increased by some beta-thalassaemia mutations (deletion and non-deletion delta beta-thalassaemia, deletions of the 5' region of the β-globin gene). Quantitative trait loci (QTL) which is the reason behind elevated HbF was demonstrated by genome-wide association have shown that genetic elements (polymorphism in *BCL11A* gene and in the HBS1LCMYB intergenic region) unlinked to β-globin gene cluster thus able to modify severity of the homozygous beta zero thalassaemia (Uda *et al.,* 2008).

The resultant eventual sequalae of the thalassaemia phenotype are mainly are influenced by these secondary genetic modifiers. A risk factor for the development of cholelitiasis in TDT and NTDT patients is the high amount of $(TA)_7$ polymorphism in the promoter region of the uridine diphosphate-glucuronosyl-transferase gene which is associated with Gilbert syndrome in the homozygous state (Galanello *et al.*, 1997; Origa *et al.,* 2009).

Apolipoprotein E ε4 allele and some HLA haplotypes are the other candidate gens for modification of the thalassaemia phenotype which tend to be genetic risk factors for left ventricular failure in homozygous beta-thalassaemia (Economou-Petersen *et al.,* 1998; Kremastinos *et al.*, 1999).

Genes that involved in iron metabolism (*C282Y* and *H63D HFE* gene mutations) has less consistent data due to their effect on iron overload being the result of iron chelation due to the regular blood transfusions and those genes that influence osseous metabolism (Longo *et al.*, 1999; Pollak *et al.*, 2000).

Other than that, a polymorphism in glutathione-Stransferase M1 gene and a higher risk of cardiac myopathy due to this iron overload in TM has been associated (Origa *et al.*, 2008). Heterozygous beta-thalassaemia has led to TI phenotype in place of the asymptomatic carrier state and majority of these most of these patients have an abundance of alpha globin genes (alpha gene triplication or quadruplication) that increased and elevated the discrepancy in the ratio of alpha and other chain synthesis (Sollaino *et al.*, 2009; Galanello & Origa, 2010).

2.2.3(b) Pathophysiology

The consequences of excess and unpaired α-globin has been reflected by erythropoiesis in individual with beta-thalassaemia (Cao & Galanello, 2010). The discrepancy between α-globin, β and γ-globin synthesis ratio is a bigger determinant of disease severity than the absence or reduced synthesis of Hb (Nienhuis & Nathan, 2012).

There is doubling in the production of α-globin chain in beta-thalassaemia trait that results in relatively normal hematopoiesis apart from mild microcytosis and hypochromia of the red blood cells. Individuals with NTDT are typically 3 to 4/ 1 of alpha to non-alpha biosynthetic ratio because the inherent ability of production of β-globin synthesis along with sparse but variable γ-globin synthesis mitigates the consequences of excess α-globin production. While marked chain biosynthetic imbalance as the underlying basis for their severe phenotype is in individuals with beta zero thalassaemia.

In addition, following synthesis, a protein complex of interaction between α-globin with its molecular chaperon which is alpha-haemoglobin stabilizing protein (AHSP) was formed before it reacts with β-globin to produce the haemoglobin tetramer

(Yu *et al.*, 2007; Weiss & Santos, 2009). The role of AHSP is to initiate folding of α-globin and prevent the formation of damaged precipitates. Microcytosis and anaemia in humans is associated with α-globin mutations that impair interaction with AHSP (Yu *et al.*, 2009). Absence of AHSP leads to amelioration of erythropoiesis in mice with beta-thalassaemia(Kong *et al.*, 2004a) suggesting that AHSP levels are a major determining factor for the phenotypical presentation of beta-thalassaemiabased on the evidence recorded (Lai *et al.*, 2006). Molecular aggregates were formed by α-globin which precipitate into inclusion bodies damaging the membrane of the cell as well as the intracellular organelles. Figure 2.2.

Other than that, the aggregated α-globin stimulate the formation of reactive oxygen species (ROS) which further harm the hydrophobic constituents of cell membrane as well as Hb and hemichromes. ROS is one of the most damaging byproduct especially for the unpaired α-chains leading to aggregation of band 3 (Nienhuis & Nathan, 2012).

The cellular apoptosis is led by the formation of α-chains inclusions in the premature stages of RBC formation and peaks in the polychromatophilic erythroblasts (Mathias *et al.*, 2000). Thus, both ineffective erythropoiesis and decreased RBC cell life which are the consequences of α-globin inclusions are reflected by the anemic state in severe Beta-Thalassaemia. Accumulations of unstable and aggregation-prone proteins are said to be causative for Parkinson's disease and Huntington's disease (Khandros & Weiss, 2010; Khandros, Mollan, *et al.*, 2012).

In order to counter the damaging effects of ROS, majority of the cells contain multiple biochemical pathways termed as protein quality control (PQC). The degradation of α-globin is carried out by the ubiquitin-proteosome system (UPS) and

the lysosome-autophagy pathways (LAP) that function in PQC. However, in severe phenotypes of beta-thalassaemiathe maximal capacity of these pathways is exceeded in the affected erythroid cells.

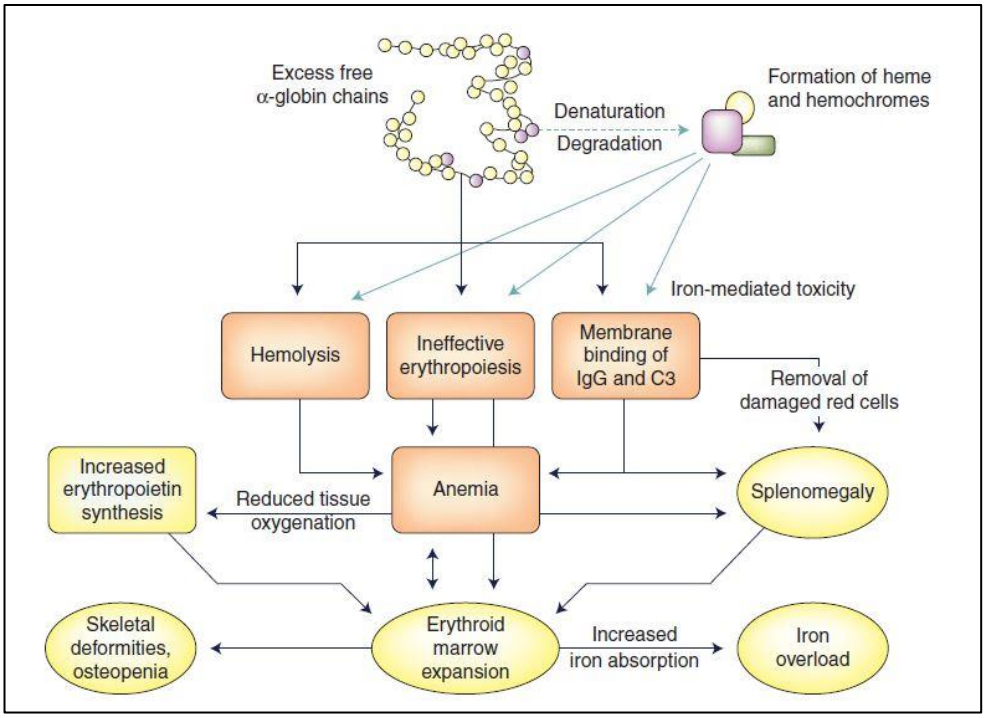

Figure 2.2 Pathophysiology of Beta-thalassaemia. Adapted from (Nienhuis & Nathan, 2012)

2.3 Haemoglobin E-beta thalassaemia (HbE/Beta-Thalassaemia)

2.3.1 Introduction

HbE/Beta-Thalassaemia genotypical combination comprises almost 50% of the severe phenotypes of beta-thalassaemia. It is characterized by a significant range of variation in clinical presentation from asymptomatic anaemia to as severe as transfusion dependent anaemia from infancy. There is still no defined protocol for the management of these patients due to the high variability of HbE/Beta-Thalassaemia and the sparsity of any long-term clinical data. The type of beta-thalassaemia mutation, the co-inheritance of alpha-thalassaemia and mutations causing excess production of fetal

haemoglobin are the genetic factors that determine the clinical severity of this disorder. The other factors that may be involved in the variability of serum erythropoietin levels is the compensatory response to anaemia, current infection or a history previous or ongoing infection (malaria), history of splenectomy and environmental influences. Customized and individualized treatment is needed for every patient and the determined management approach should be followed up and assessed over a period of time. This is due to the condition showing marked variability owing to the inherent instability of the clinical phenotype of HbE/Beta-Thalassaemia (Olivieri, Pakbaz & Vichinsky, 2011b).

2.3.2 Epidemiology

Those patients diagnosed with severe beta thalassaemia are 50% represented by individuals with HbE/Beta-Thalassaemia (Chen *et al.*, 1996a; De Silva *et al.*, 2000a; Weatherall & Clegg, 2001; Premawardhena *et al.*, 2004a; Modell & Darlison, 2008). India, Bangladesh and throughout Southern Asia, particularly Thailand, Laos and Cambodia have the highest prevalence due to the population there more predisposed to the inheritance of homozygous alleles for both haemoglobin E (HbE) and beta-thalassaemia (Weatherall & Clegg, 2001).

This disorder has become an increasingly severe public health problem affecting 3000 children in every 100000 (Flint *et al.*, 1998). The average inheritance of gene stands at about 4% for beta-thalassaemia and for Hb E, meaning 1000s of people are affected in southern China (Angastiniotis & Modell, 1998). Despite being one of the rarely reported condition, HbE/Beta-Thalassaemia is slowly becoming one of the most commonly detected form of beta-thalassaemia in the routine screening programmes in North America and Europe. In Indonesia and Sri Lanka, this disorder is common

(Weatherall & Clegg, 1996, 2001; Angastiniotis & Modell, 1998; Rees *et al.*, 1998; Lorey, 2000; Weatherall, 2010).

2.3.3 Pathophysiology

The co-inheritance of a beta-thalassaemia allele from one parent and its structurally isomeric form HbE from the other results in HbE/Beta-Thalassaemia. Substitution in codon #26 of G → A of the beta-thalassaemia globin gene results in the formation of Haemoglobin E that produces structurally unstable haemoglobin and activates a cryptic splice site resulting in abnormal messenger RNA (mRNA) processing. The abnormally spliced mRNA is rendered non-functional when a new stop codon is generated due to this situation (Orkin *et al.*, 1982).

Hence, a milder form of beta-thalassaemiais produced due to the synthesis of haemoglobin E at a reduced rate. Many factors such as decreased β chain formation that results in globin chain discrepancy, ineffective erythropoiesis, apoptosis, oxidative damage and temporal reduction in red blood cell survival are related to the pathology of HbE/Beta-Thalassaemia (Pootrakul *et al.*, 2000; Datta *et al.*, 2006). The instability of HbE is a relatively minor factor when you consider the entire pathophysiology of HbE/Beta-Thalassaemia, however, in cases of recurrent febrile episodes this instability causes accelerated hemolysis leading to rapid deterioration of patient condition (Jetsrisuparb *et al.*, 2006).

2.3.4 Phenotypic heterogeneity of Haemoglobin E-beta-thalassaemia

Through the study in a clinic-based populations in Sri Lanka, relatively lesser cases were observed than expected based on Hardy-Weinberg equation, probably reflecting the mild and variable clinical course of some cases of this disorder (De Silva *et al.*, 2000a).

A modified “Natural history study” of this condition in children of Sri Lanka elucidated the inherent instability of this genotype which causes variability in the severity of anaemia and erythroid expansion during the first decade of life. This is because phenotype of this disorder is reported as unstable (Olivieri, Pakbaz & Vichinsky, 2011a).

The mean difference in haemoglobin concentration was only around 1 to 2 g/dL between the mildest and severest levels. In patients sustained on daily or "on demand" transfusions, concentrations of pre-transfusion steady-state haemoglobin (mean 7.0 g/dL) were clinically similar (mean 6.1 g/dL) to those in patients who were never initiated on routine transfusions (Premawardhena *et al.*, 2005).

2.3.5 Clinical severity categories of Haemoglobin E-beta-thalassaemia

The categorization of patients into “severe” and “mild” spectrum of the disease process is based on the genetic mutation and environmental factors rather than the severity of HbE/Beta-Thalassaemia (Premawardhena *et al.*, 2005; Sripichai *et al.*, 2008a). A study in Sri Lanka, involving 109 patients (aged one to 51 years) categorized them into five classes of severity ranging from very mild to very severely affected patients (Premawardhena *et al.*, 2005).

Approximately one fifth of patients were transfusion-independent while the remainder were kept on transfusion, either frequently or intermittently (on demand). There was a lack of clear understanding in many patients as to why or whether transfusions were required (Olivieri & Brittenham, 1997; Premawardhena *et al.*, 2005; Olivieri *et al.*, 2008).

2.4 Alpha Haemoglobin Stabilizing Protein (AHSP)

2.4.1 Introduction to AHSP

Alpha haemoglobin stabilizing protein (AHSP), is also known as an erythroid differentiation-related factor (EDRF), or quite simply, erythroid-associated factor (ERAF). It has been identified as an erythroid specific protein that binds with free α-globin and stabilizes it in-vitro and in-vivo. The combination between α-globin and AHSP inhibits the highly cytotoxic ROS produced due to different interactions of free α-haemoglobin in the body. It prevents the precipitation of highly unstable and toxic free α-globin chains, which aggregate in erythroid precursors, damaging the cell membrane and eventually triggering cell death (Costa & Favero, 2011). AHSP is known to be in abundance in the late stages of erythroid precursors and its expression kinetics has been determined to draw parallels to that of α-globin (Mahmoud *et al.*, 2015).

2.4.2 Genetics, molecular structure and expression

The human *AHSP* gene is found at chromosome 16 (16p11.2) and extends over 952 bases with three exons, two introns and one untranslated region (UTR) at the end of exon 3. The initiation of the translation and the termination codons are found on the exon 2 and 3 respectively (Figure 2.3).

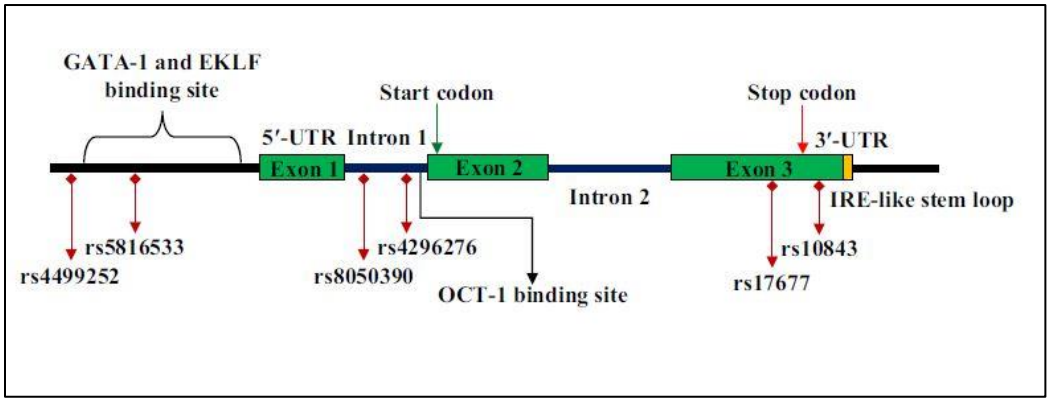

Figure 2.3 Structure of *AHSP* gene and common polymorphisms. Adapted from (Che Yaacob *et al.*, 2020)

The *AHSP* gene encodes a small protein molecule by the same name (11,84 kDa) with 102 amino acids expressed in the marrow at the highest level. AHSP protein comprises about 70% of α-helices and can occur in cis and trans isomeric forms which can be differentiated from loop 1 region arrangements between helix 1 and proline-contributing helix 2 (30th residue) (Santiveri *et al.*, 2004) (Figure 2.4).

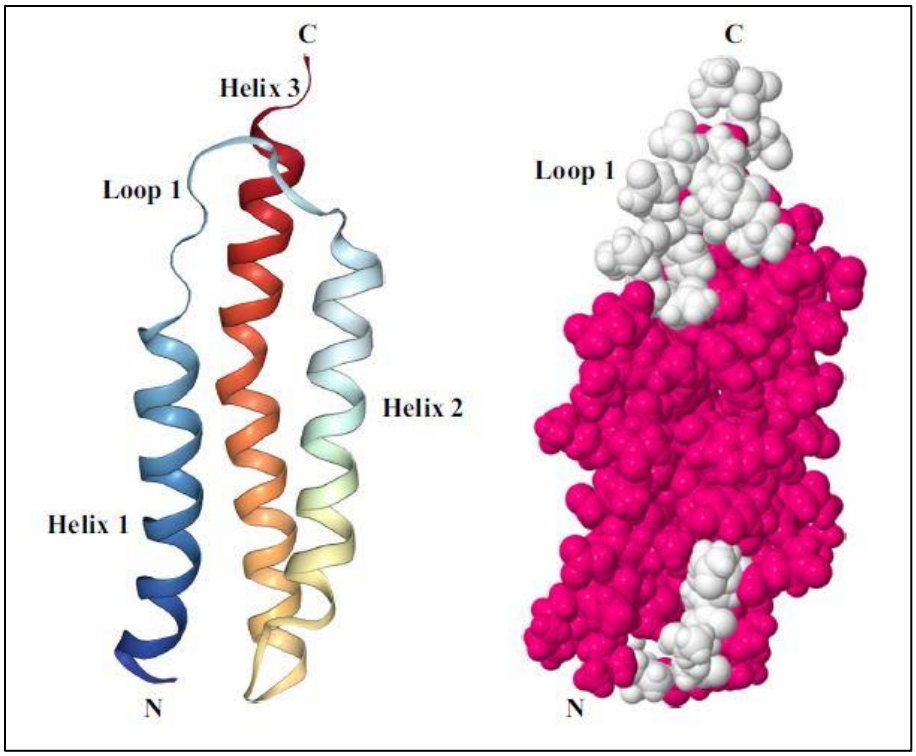

Figure 2.4 Molecular structure of *trans* form of AHSP. Adapted from (Che Yaacob *et al.*, 2020)

A variety of transcription factors have been found to regulate the expression of the *AHSP* gene, including GATA-1 (Gallagher *et al.*, 2005), Oct-1 (Gallagher *et al.*, 2005), EKLF (Pilon *et al.*, 2006; Keys *et al.*, 2007), STAT3 (Cao *et al.*, 2014) and NFE2 (Zhao *et al.*, 2010). When the erythroid transcription factor NFE2 binds to *AHSP*, it triggered the transcription process and the occupancy of GATA-1 in NFE2-deficient

cells decreased which provides an interesting insight into the significance of NFE2 in *AHSP* expression pattern.

On activation of STAT3, increased *AHSP* expression was seen in K562 cells and vice versa (Cao *et al.*, 2014). It was found in a twin heritability study (Lai *et al.*, 2010) that the majority (46%) of the *AHSP* expression was controlled by multiple genetic heritability. Iron-responsive element (IRE) like stem-loop structure at the 3′-UTR of *AHSP* mRNA regulated *AHSP* gene expression (Dos Santos *et al.*, 2008).

2.4.3 Function and mechanism of action

AHSP's main function is to reversibly bind the free α-globin to stabilize the structure, and to reduce its chemical reactivity. AHSP cannot bind to β-globin nor to tetrameric HbA1 ($\alpha_2\beta_2$). This controlled relationship prevents α-globin from being aggregated and precipitated in vitro and in vivo, so α-globin remains available to form HbA1 ($\alpha_2\beta_2$). Both AHSP and β-globin have the same binding sites for α-globin, but β-globin has a higher binding affinity to α-globin than AHSP, so β-globin will cause AHSP to be displaced from AHSP–α-globin and HbA1 ($\alpha_2\beta_2$) molecules (PINHO *et al.*, 2008; Krishna Kumar *et al.*, 2010; Turbpaiboon & Wilairat, 2010).

The N-terminal part of the α-globin H-helix is involved in the interaction with the AHSP and the C-terminal part is essential for heme interaction, both of which make the α-globin stable (Domingues-Hamdi *et al.*, 2014). Another study revealed that AHSP dislodges from the α-globin subunit during the reduction of the number of these globin chains and starts adhering to β-globin to form a functional tetramer (Kiger *et al.*, 2014). α-globin auto-oxidation by introducing strain into the proximal heme pocket when β-globin is in limited number is promoted by Proline 30th residue of AHSP (Gell *et al.*, 2009; Dickson *et al.*, 2013). The subunit complexes AHSP–α-globin do not engage in