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## Genetic Background of $\beta$ Thalassemia Modifier: Recent Update

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### Abstract

Thalassemia has become major health problem among developing countries. Genetic background which contains enormous mutations and variations have lead in clinical problem differences.

The genetic basis of thalassemia, beta specifically, is mutations of the gene encoding the  $\beta$  chain of the hemoglobin (Beta-Globin, HBB). However, today it is known that abnormalities in this gene do not necessarily determine the clinical appearance of  $\beta$  thalassemia patients. Currently there are several genetics and non genetics factor that are influence clinical manifestation such as the type of  $\beta$  globin, gene mutation, co-inheritance with  $\alpha$  thalassemia and HPFH and also non genetic factor that worsen the clinical symptom i.e infection, nutritional status, and oxidation state such as iron overload. Several genetic studies are still notable to explain the cause of inconsistency between genotype and phenotype of  $\beta$  thalassemia.

A set of genes that can modify the primary  $\beta$  thalassemia disorder has been founded. Secondary modifier contains genes that have been associated with elevated levels of HbF or continuous production of  $\gamma$  chain and improvement ratio of  $\alpha$  / non  $\alpha$  globin chain. The genes involved are HBA, HBG, BCL11A, HBS1L-MYB and other cofactor genes regulating erythropoiesis. Tertiary genetic modifier comes from other genes related to the disease severity including iron metabolism, redox activity, and clinical complications. The review aims to provide the latest updates regarding the known  $\beta$  thalassemia modifier genes and some other genes involved in the changes of the clinical manifestations.

**Keywords:** thalassemia;  $\beta$  thalassemia; genetic modifiers; BCL11A; HBS1L-MYB; XmnI

### INTRODUCTION

Thalassemia is a hereditary hemolytic disease with the highest prevalence and incidence in the world. It is one of the most serious health problems given that thousands of children suffer from it each year. Thalassemia is most prevalent in the areas known as the Thalassemia Belt, such as the Mediterranean, Middle East, Southern Asia, China Peninsula, Southeast Asia, and Pacific Islands<sup>1</sup>. Recent reports suggested that Thalassemia has rapidly spread to America, Europe, and Australia due to migration and inter-marriage of different ethnic groups. The World Health Organization (WHO) estimates that 7% of the global populations (80 – 90 million people) are carriers of  $\beta$  thalassemia where most of them from developing countries.

The high frequency of Thalassemia in these regions is strongly associated with the spread of *Plasmodium falciparum*. Of the population who are endemic with malaria, about 3 – 40 % carries the variant hemoglobin, with thalassemia major prevalence at about 0.3 – 25 per 1000 births. The literature suggests that from the numbers, only about 200,000 patients with the major symptoms of Thalassemia that are registered and receive regular governance<sup>2</sup>. The data from Indonesia reported that  $\beta$  thalassemia carriers were 3 - 5% population and in some areas this numbers even reaches 10%. It is estimated that there will be 2500 children born with thalassemia annually. This number is far below the real number found in the population. This discrepancy

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is thought to be caused by several factors including the variety of clinical features that range from extremely mild symptoms that do not require transfusion to the severe ones that need regular transfusion.<sup>3</sup>

## Molecular Pathophysiology

### 1. Primary Modifier of $\beta$ thalassemia

It has been shown that clinical heterogeneity in  $\beta$  thalassemia primary is caused by variations of genetic lesions in  $\beta$  globin gene. Patient severity and the amount of produced globin protein are directly associated with a mutation on the  $\beta$  globin gene.<sup>4,5</sup> More than 300 point mutations in  $\beta$  globin gene have been identified and deletion is very rare. However, genotype variability in  $\beta$  globin gene only is often not enough to explain the different phenotypes when the patients show the same genotype<sup>6</sup>. Wahidiyat, 2009 had reported a family who had two  $\beta$ thalassemia/HbE children, one of them need 6-8 times transfusion per years, while the other does not require transfusion. The hemoglobin and DNA analysis showed the same mutation,  $\beta^+$  severe thalassemia/HbE was present in both children. This case shows how patients with the same genetics and non genetics (environmental) factors may show different clinical manifestations.<sup>3</sup>

$\beta$  thalassemia occurs due to the disappearance of the  $\beta$  globin chain ( $\beta$  thalassemia<sup>0</sup>) or the disruption of  $\beta$  chain ( $\beta$  thalassemia<sup>+</sup>) on blood globin structure. In  $\beta$  thalassemia, there is an imbalance of production between the globin  $\alpha$  chain and globin  $\beta$  chain that would normally form A hemoglobin (Adult).<sup>7</sup> The disruption in the synthesis of  $\beta$  globin in  $\beta$  thalassemia patients lead to an excess of the globin  $\alpha$  chain, so there is  $\alpha$  chain precipitation inside the red blood cell precursor in the bone marrow and also in the derivatives of peripheral circulation. The erythroid nuisance induces an ineffective erythropoiesis shown by the proliferation of bone marrow cells and extra medullary erythropoiesis.<sup>8,9</sup> Furthermore, products like heme, hemin, hemichrome, and loose iron can cause a pathological change in red blood cells due to oxidative damage of the cell membrane that leads to apoptosis. A further study suggested that the erythroid precursor of a Thalassemia patient showed 3-4 times higher apoptosis level than the erythroid in a normal person.<sup>10</sup> Mechanism, able to reduce the  $\alpha$ /non  $\alpha$  chain imbalance is the co-inheritance with a thalassemia, continuous production of  $\gamma$  chain which binding the excess of  $\alpha$  chain result in a persistent fetal haemoglobin (HbF) production

The clinical phenotypes of  $\beta$  thalassemia patients vary depending on the requirement of blood transfusion. The phenotype is associated with the degree of imbalance and the surplus of the globin  $\alpha$  and  $\beta$  chain. This clinical spectrum involves many factors, including the type of mutation in the  $\beta$  gene as a primary modifier, a secondary modifier that leads to the improvements of the balance ratio of  $\alpha$  and  $\beta$

globin chain, and also a tertiary modifier which is both genetic and non-genetic that ameliorates or aggravates with the disease appearances and complication.<sup>11</sup>

### $\beta$ Globin Mutations.

Clinical variability of  $\beta$  thalassemia patients correlates with the mutation type contained in the globin  $\beta$  gene. Allele classification is distinguished into two major classes that are a severe allele with total absence of the globin chain, and the mild allele which produces a globin chain that is inheritable. The alleles are notated as  $\beta^0$  and  $\beta^+$ , respectively. Most of the mutations that cause these types are point mutations. Another type involves a deletion.<sup>12</sup>

Today, more than 300 unique mutations have been well characterized. There are four known major mutants of  $\beta$  thalassemia: a promoter mutant, RNA splicing, RNA capping/tailing mutant, and a translation mutant.<sup>6</sup> Another form of molecular abnormality is a frameshift mutation that can transform the reading frame, thus resulting in a short and unstable polypeptide or the formation of a codon stop (nonsense mutation) that results in a premature termination of translation process.<sup>13</sup> A variation of these kind mutations will affect on the first transfusion time, transfusion requirements, and clinical appearances in the patient's life.<sup>14</sup>

The mutation that interferes with the transcription process can happen in the 5' untranslated region (5' UTR) of the globin  $\beta$  gene as well as in the proximal CACC box. An example of the mutation is -90 C>T, -88 C>T, -88 C>A. Moreover, the mutation can also happen in the TATA box region such as -31 A>G and -30 T>A. Generally, the mutations interfere with the production of the globin chain on a basic level thus suggests a picture of mild clinical diagnosis of  $\beta^+$  or even silent  $\beta^{++}$ .<sup>15</sup>

The mutation that involves the RNA splicing process can happen on the splice donor site such as IVS2-2-T or splice acceptor site such as IVS2-850 G>T that leads to a clinical picture of severe Thalassemia ( $\beta^0$ ) due to non-formation of the normal RNA. The mutation that is located between those areas can lead to the decrease of varied RNA which raises the Thalassemia phenotype from mild to severe. One of the mutations is IVS2 -5 G>T. A variant structure of HbE (Cd26 GAG>AAG) is the general form in the Southeast Asian population which can result in the mild type ( $\beta^+$ ) Thalassemia phenotype because it could activate cryptic splicing of the GTGGTGAGG donor in codon 24-27 of exon 1. The activation which causes the normal production of HbE is reduced so the clinical manifestation of Thalassemia can occur.<sup>16</sup>

The mutation that involves the start codon can interfere with the initiation of the transcription process and results in the  $\beta^0$  phenotype; for instance, the ATG>AGG mutation. The mRNA elongation process can be interrupted by nonsense mutation like Cd17 AAG>TAG or a frameshift mutation like Cd41/42 -TTCT. These mutations cause a premature termination of the mRNA and result in a severe Thalassemia phenotype ( $\beta^0$ ). Some of the  $\beta^+$  and  $\beta^{++}$

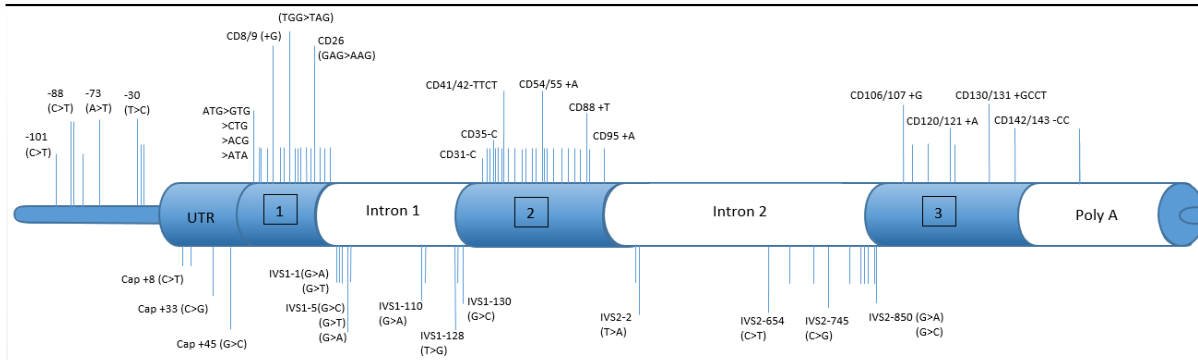


Figure 1.  $\beta$  Globin Gene and Common  $\beta$  Thalassemia Mutation

$\beta$  globin gene is built from 3 exons and 2 intervening sequences (introns). The majority of  $\beta$  gene mutations are located in front of the gene and predominantly by point mutations. Upstream mutations may affect TATA box region and 5' UTR including start codon ATG. Exon 1, exon 2 and intron (IVS/intervening sequences) 1 are the most prevalence mutations in  $\beta$  gene, while the rest are less common.

phenotypes are caused by a mutation in the polyadenylation region (AATAAA) at 3' position in the end of the globin  $\beta$  gene that can destabilize the mRNA chain. An example of this mutation is AATAAA>CATAAA.<sup>17</sup> The full set of common mutations in  $\beta$  thalassemia is depicted in figure 1.

Some of the mutants show a dominant reduction nature.<sup>13</sup> On the mutant that produces a premature termination codon (PTC) of mRNA that is caused by the frameshift mutation or nonsense mutation, this mRNA will be degraded by nonsense-mediated mRNA decay (NMD) so the presence of the mRNA is at a minimum level.<sup>18</sup> If the PTC mutation occurs in exon 3 of the globin  $\beta$  gene, NMD does not function well and abandons the chain, so the mRNA can be translated and produces variant globin protein that can cause a precipitation in the erythroid precursor.<sup>6</sup>

## 2. Secondary Modifier of $\beta$ thalassemia

The clinical pictures of Thalassemia that are caused by a primary mutation of the  $\beta$  globin can be modified by another gene outside the  $\beta$  globin gene. This secondary genetic factor modifies the Thalassemia phenotype by repairing the imbalance of the globin chain and the surplus of globin chain. HbF which is normally expressed in the fetus period is able to modify the clinical picture by binding the surplus of  $\alpha$  chain on  $\beta$  thalassemia.<sup>11,19</sup> The surplus of an  $\alpha$  chain on  $\beta$  thalassemia can cause a precipitation of the erythrocyte membrane as a hallmark of the disease that can be modified by some conditions like a coinheritance with an  $\alpha$  mutation and the increase of HbF.<sup>20,21</sup>

### $\alpha/\beta$ Globin Ratio Modifier.

It has been reported that homozygous  $\beta$  thalassemia or a heterozygote compound that carries a deletion allele of  $\alpha$  gene show less severe phenotype. The mechanism of this modification effect depends on the severity level of the mutation allele type on the  $\beta$  gene and the number of functionalities of the  $\alpha$  gene.<sup>15</sup> The coinheritance from a single deletion of the  $\alpha$  gene on  $\beta$  thalassemia has a minimum impact while the deletion of two  $\alpha$  genes shows a less severe phenotype.<sup>22</sup> The occurrence of an  $\alpha$  gene deletion

causes a balance between  $\alpha$  and  $\beta$  chains on  $\beta$  thalassemia and the patient recovers. With a heterozygote  $\beta$  thalassemia patient, a single deletion of the  $\alpha$  gene shows an absence of hypochromic microcytic blood pictures, while the addition (extra copy) to the  $\alpha$  gene causes a more severe clinical picture in  $\beta$  thalassemia patients. Some of the heterozygote  $\beta$  thalassemia patients with a triplication of  $\alpha$  genes ( $\alpha\alpha\alpha / \alpha\alpha\alpha$ ) show a phenotype spectrum from asymptomatic to intermediate Thalassemia.<sup>23</sup> Another study also reported that quadruplication of  $\alpha$  genes in simple heterozygosis and in carriers of suspected silent  $\beta$  thalassemia, may result in symptomatic thalassemia.<sup>24</sup> The recent reports shows that this kind of genotype constitution is not rare in case of thalassemia intermedia.<sup>25</sup> The balance abnormalities of  $\alpha$  and  $\beta$  chains on  $\beta$  thalassemia are also related with major  $\alpha$  globin gene regulator. Homozygous deletion on these regulator may produce Hb H disease appearance,<sup>26,27</sup> but in coinheritance with  $\beta$  thalassemia could modify  $\alpha$  and  $\beta$  chain ratio, impacting to a better condition.<sup>28</sup>

### Increasing of HbF as Modifier

The ratio of  $\alpha$  and  $\beta$  chains on  $\beta$  thalassemia can also be modified by an increase of HbF production. Though in normal adults the production of HbF is stopped when they were born, the HbF is still minimally produced ( $\pm 1\%$ ). In the case of  $\beta$  thalassemia, HbF is relatively increased due to the selective survival from the erythroid precursor that synthesizes the  $\gamma$  chain. A normal adult has 0.5 – 1% of HbF which is concentrated at F-cells (3-7% of erythrocyte total). However, the  $\beta$  thalassemia patients have a different ability in terms of the number of synthesized  $\gamma$  chains.<sup>13</sup>

The enhancement of HbF in the adult can occur in the cases of Hereditary Persistence of Fetal Hemoglobin (HPFH) and through the Quantitative Traits Locus (QTL). The deletion of seven areas within the locus of  $\beta$  globin has been characterized and associated with HPFH. These HPFH deletions show the enhancement of HbF by 30% of total hemoglobin expressed on all of the erythrocytes (pan-cellular HbF).<sup>29</sup> The basis mechanism of the

abnormality may be caused by the inability of normal  $\gamma$  globin diversion due to juxtaposition from the enhancer distal element in the proximal area of the  $\gamma$  globin gene as a result of the deletion.<sup>17</sup>

Different from the deletion HPFH type, where both  $G\gamma$  and  $A\gamma$  genes run into overexpression, non-deletion HPFH expresses only a single globin  $\gamma$  gene. The identification of a point mutation in a non-deleted HPFH case is 5 mutation types that have been found on GC rich -200  $G\gamma$ , -202 C>T, -198T>C, -196C>T, -195 C>G from  $A\gamma$  gene.<sup>30</sup> HPFH expressed only a single globin  $\gamma$  chain is limited to *XmnI*<sup>G $\gamma$</sup>  polymorphism. Pathomechanism of HbF enhancement on the non-deletion HPFH is still a controversial topic. The affinity enhancement of Spl protein is reported to have a correlation with the HbF enhancement in the case of HPFH on several point mutations that are -202 C>G, -198 T>C and -195 C>G.<sup>31</sup> Recent studies found that this kind of mutation is a *de novo* binding site from the Stage Selector Protein (SSP) which is a protein complex that causes an adhesion with the proximal promoter, while -198T>C creates a novel CACCC box in the globin  $A\gamma$  promoter.<sup>32</sup>

In addition to HPFH, the increased production of HbF involves genes that are included in the Quantitative Traits Locus (QTL) of HbF Inducer. There are at least three main loci that are associated with the increased production of HbF and affect the phenotype of  $\beta$  thalassemia patients. Those loci are Gy promoter, chromosome 6q, and chromosome 2.<sup>11</sup>

### Quantitative Traits Locus (QTL) of *XmnI*, *BCL11A*, and *HBS1L-MYB*

A -158 C>T point mutation on Gy promoter, noted as *XmnI* locus, is known to play a role in the relative overproduction of HbF. In a non-anemic population in Northern Europe, *XmnI* inside of the locus is correlated with the increase of HbF on the level of 13-32 % F cells.<sup>33</sup> Another study suggested that the *XmnI* locus played a role in determining the prognosis in Sickle Cell Disease.<sup>34</sup> A more detailed study from Lettre et al.<sup>35</sup> suggested that SNP rs7482144 (*XmnI* locus) explained the 2.2 % increase in HbF production variety in an SCD case. In some cases of  $\beta$  thalassemia, the SNP suggests a Linkage Disequilibrium with mutations like 6 -A codon and 8 -AA codon that is consistently associated with the increase of HbF, resulting in an intermediate Thalassemia phenotype.<sup>11</sup> Another study suggests that the *XmnI* genotype (+/+) can be used as a phenotype predictor of  $\beta$  thalassemia cases.<sup>36</sup> However, the role of SNP rs7482144 for the relative increase of HbF is inconsistent in normal adults and heterozygote Thalassemia.<sup>37</sup>

A central role of SNP rs7482144 has been confirmed in Genome Wide Association Study (GWAS). In a European study, the traits variance of the locus is 10.2 %.<sup>38</sup> The *XmnI* locus is further reported as having a role in the increase of HbF in intermediate Thalassemia.<sup>36</sup> In India, the genotype (+/+) suggests a meaningful correlation with the clinical occurrence of  $\beta$  thalassemia patient,<sup>39</sup> while

the population study on heterozygote Thalassemia patients in China suggests that the *XmnI* locus is associated with the increase of 9 % HbF and 13 % of F-cell.<sup>40</sup> However, in the African population in America with SCD, SNP rs10128556 is more strongly correlated than the rs7482144 / *XmnI*, indicating that the *XmnI* locus in this ethnic group is not a causal variant.<sup>41</sup> The data from Indonesia reported that genotypes (-/-) and (+/) of the *XmnI* locus is not related with the absolute amount of HbF in HbE/  $\beta$  thalassemia patients.<sup>42</sup> The same group reported that  $\beta$  thalassemia mutation type is not a factor that influence clinical manifestation, apart from the IVS1-nt 5 (G>C) that is related to severe clinical features, co-inheritance of a globin deletion type 3,7 kb (co/o-) is related to mild clinical manifestation, while  $\alpha$ -globin chain triplication did not exist both mild and severe groups and therefore its relation with clinical manifestation could not be assessed. Study also showed that *XmnI* heterozygote polymorphisms (polymorphism in-150<sup>o</sup>y globin gene, C>T) were found both in the mild and severe groups, therefore this kind of polymorphism is not a factor that influences clinical manifestations. From three SNPs, only bg200 was related to clinical manifestations the CC genotype at SNP bg200 is related to low HbF level and severe clinical manifestations, and have 4.15 times greater risk of having severe manifestation than those without.

In addition to the  $\beta$  gene cluster, a study of Asian-Indian patients with  $\beta$  thalassemia that was accompanied by a sample segregation with rising HbF, found that the locus of the 6q23-q24 chromosome is correlated with the increase of HbF. The locus shows an association where the genome-wide linkage analysis (GWLA) is meaningful in the less severe  $\beta$  thalassemia phenotype.<sup>11</sup> Further studies suggest that the areas along 1.5 Mb in 6q23-q24, there is an intergenic area of *HBS1L* and *MYB* genes.<sup>38,43,44</sup> The locus is associated with ~19 % of the natural variety of the population in Europe.<sup>38</sup> The research found that 3 SNPs were the main markers that have the highest correlation to HbF and F-cells level: rs9399137, rs5209090, and rs6929404. A Population study in China and African-American suggest that the two main SNPs correlated with this are rs9399137 and rs7775698.<sup>45</sup> Homozygosity for the 3-bp deletion in *HMIP* and heterozygosity for the HbH  $\alpha$ -thalassaemia mutation in *HBA2* were also reported as the two genetic determinants found only in the asymptomatic of FSC8 homozygote twins and not found in other FSC8 typical transfusion dependent patients.<sup>46</sup>

With the marker from *BCL11A* rs4671393, *HBS1L-MYB* rs9399137, rs28384513, and rs4895441, and rs7482144 *XmnI* locus, Lettre et al.<sup>34</sup> suggested that SNPs is responsible for >20 % of HbF phenotype varieties and is associated with a better clinical prognosis. Genetic variety in the area of *HBS1L-MYB* is significantly different between the individual with high HbF and normal individuals.<sup>41</sup> The basis mechanism from the situation is possibly related with the role of *MYB* in the erythropoiesis process. *MYB* gene overexpression inhibits the  $\gamma$

globin in erythroleukemia cells. Furthermore, research data suggests that the low level of MYB protein in the initiation of erythropoiesis is correlated with rising HbF levels.<sup>47</sup>

The BCL11A locus in chromosome 2 is known to be associated with the increase of HbF based on the findings that the gene is responsible for the Hb transition mechanism.<sup>48,49</sup> Fine mapping on the BCL11A locus in the African-American population with SCD found three independent SNPs that are related to HbF. Haplotype analysis (based on SNP) suggests that the haplotype in this locus describes a better phenotypic variance on the cumulative amount than the three SNPs when taken individually (18.1 vs. 14.7 %).<sup>41</sup>

The findings are confirmed in the normal population of Chinese and Thai descent carriers of  $\beta$  thalassemia<sup>50</sup> and patients with sickle cell anemia from the USA, Brazil, and the UK.<sup>35</sup> It was suggested that the SNP rs766432 minor allele (C) has the strongest association with the increase of HbF and F-cell in the Chinese, Thai, and African-American populations<sup>50</sup>, while the SNP rs11886868 is associated with the increase of HbF in the Caucasian population in lower levels.<sup>51</sup> The study on another population in China suggested that rs11886868 has a strong association with the increase of HbF in Thalassemia patients<sup>21</sup>, while in Thailand, rs766432 on BCL11A has a strong association to the increased level of HbF<sup>52</sup>, as well as in Indonesia.<sup>53</sup> A very recent study found that the LRF/ZBTB7A transcription factor occupies fetal  $\gamma$ -globin genes and maintains the nucleosome density necessary for  $\gamma$ -globin gene silencing in adults, and that LRF confers its repressive activity through a NuRD repressor complex independent of the fetal globin repressor BCL11A.<sup>54</sup>

Quantitative Traits Locus of HBG, BCL11A and HBS1L-Myb in ameliorating  $\beta$  thalassemia through elevating HbF have been known and ready to implement in thalassemia management. The Thalassemia Severity Score web-tool (<http://tss.unica.it>) is available now to calculate and predict the severity of clinical phenotype based on prominent marker;  $\beta$  type mutations,  $\alpha$  deletion, HBG2:g.-158C>T, BCL11A rs1427407, BCL11A rs10189857, and HBS1L-MYB rs9399137. Validation of the model was conducted by Danjou and colleagues.<sup>55</sup>

### Element factors of EKLf, GATA, GDF11, GDF15, and AHSP

The Kruppel-like factor 1 (KLF1), a key factor for primitive and definitive erythropoiesis has been reported to have significant rule in ameliorating clinical phenotype of  $\beta$  Thalassemia. Individual with a dominant missense mutation in the KLF1 zinc-finger domain has ineffective erythropoiesis picture leading to congenital diserythropoietic anemia (CDA).<sup>56</sup>  $\beta$  Thalassemia patient who has this kind of mutation can be fall into severe condition. Conversely, there are sequence alterations that result in HFPFH may actually ameliorate the disease severity. A high HbF levels of up to 40% of total hemoglobin (Hb) was already

found in individual with compound heterozygotes for class 2 and 3 *KLF1*.<sup>57</sup> Recent study also reveal that class 2 or 3 *KLF1* variants are associated with moderately increased levels of HbA<sub>2</sub>, making attention to  $\beta$  Thalassemia carrier screening program.<sup>58</sup>

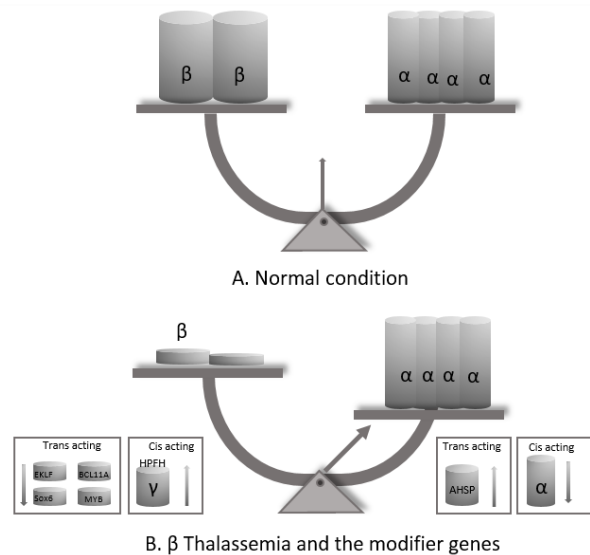


Figure 2. Normal and  $\beta$  thalassemia condition

A. Erythrocytes contains normal composition of globin chain; two  $\beta$  globin chains pair with four  $\alpha$  globin chain. B. Mutation in  $\beta$  globin chain disrupt the balance of  $\alpha$ /non  $\alpha$  ratio. Improvement of the ratio could be achieved by decreasing trans acting factors (KLF1, Sox6, MYB, and BCL11A) or accelerating cis factor including HFPFH and  $\gamma$  globin chain. In another side, decreasing  $\alpha$  globin chain through deletion or increasing AHSP activation would be benefit for  $\alpha$ / $\beta$  ratio.

Cofactor FOG-1 (friend of GATA-1) primarily regulates erythropoiesis and megakaryocytopoiesis. Mutations in the FOG-1 binding sites of its N-terminal zinc finger are responsible for dyserythropoietic anemia with thrombocytopenia, facing to severe phenotype.<sup>59</sup> It has been shown that elevated Epo production is associated with high levels of Jak2 phosphorylation. This kind action promotes escalation of erythroid progenitors which contribute to extramedullary hematopoiesis. Literature review of these studies proposed that acute administration of a Jak2 inhibitor (JAK2i) could reverse the splenomegaly in thalassemic patients avoiding the need for splenectomy.<sup>60</sup> Other findings were also interesting. Growth differentiation factor 11 (GDF11), a factor responsible for rejuvenation of stem cell is promising. Ongoing clinical trial is showing amelioration of the anemia in NTDT patients and a potential reduction of the transfusion regimen in patients affected by  $\beta$ -thalassemia major.<sup>60</sup> Another factor is GDF15. An abundant expression of GDF15 inhibited hepcidin expression, contributing to iron overload process in thalassemia syndrome.<sup>61</sup> Reducing GDF15 factor may decrease toxicity of iron and contribute to clinical improvement.

Another genetic determinant reported as having a link with the milder phenotype of  $\beta$  Thalassemia is the  $\alpha$ -hemoglobin-stabilizing protein (AHSP), the active molecular chaperon which binds to the free  $\alpha$  chain. AHSP protein can act as a stabilizer for the  $\alpha$  chain at the time of globin chain binding and prevents a precipitation.<sup>62</sup> Mice knocked-down on AHSP protein have an abnormal red blood cell production and showed shorter lifespan, allegedly caused by a relative surplus of unused globin chains. Furthermore, the phenotype was aggravated upon testing with the intermediate Thalassemia genotype, corresponding to the loss of AHSP.<sup>63</sup>

By using K562 cells, it was suggested that AHSP can be a candidate genetic modifier in thalassemia patients.<sup>64</sup> It was also reported that an individual in Southern Asia with an AHSP mutation homozygote (Val56>Gly), in the initial year of life shows a clinical syndrome similar to a Thalassemia patient.<sup>65</sup> Research on Thalassemia patients suggested that the effect of mutations or polymorphisms in AHSP is still controversial. Previous studies suggested that the mutation on the gene is not significantly associated with the severity of the  $\beta$  thalassemia patient population in China<sup>66</sup> and Thailand.<sup>67</sup>

### 3. Tertiary Modifier of $\beta$ thalassemia

The course of the Thalassemia clinical syndrome can be affected by many factors including genetics that are associated with a blood component, genetics outside of the blood component, or non-genetic conditions.<sup>68</sup> The oxidative stress in Thalassemia patients occurs due to hemoglobin instability, the surplus of iron, and hemolysis. In normal conditions, red blood cells degrade the reactive oxygen species (ROS) through the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities.<sup>69,70</sup> In the thalassemia cell, there is heme release that induces the amplification of the oxidative pathway. The excess iron can also act as a catalyst of fat peroxidase. Moreover, the hypochromic condition can cause an acceleration of the oxidative process due to a lessening of buffer protection by hemoglobins. It has been reported that the accumulation and auto-oxidation from the unpaired globin  $\alpha$  chain will produce superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) that leads to accelerated apoptosis and ineffective erythropoiesis.<sup>71</sup>

Several studies reported that the oxidative activity is associated with the level of iron levels in the body.<sup>72</sup> By using a curcuminoid as an antioxidant, Kalpravidh and colleagues suggested that malondialdehyde, superoxide dismutase, and GPx enzyme significantly decrease with 12 months of therapy. In these studies, it was reported that curcuminoid can also be an alternative iron chelator that can decrease the ferritin levels. The excess accumulation of iron can increase the free radicals in the circulation. An individual's ability to parse is controlled by a genetic mechanism that is appropriate with the involved enzyme.<sup>73</sup>

Glutathione and Catalase are two enzymes that initiated the regulation of the free radicals.

Glutathione S transferase (GST) is a superfamily enzyme from multifactorial isoenzymes that play role in detoxification and excretion of toxic molecules. Evidence suggests that the expression level of GST is a key factor in determining the cell sensitivity in a wide spectrum towards the toxic chemicals.<sup>74</sup> The enzyme is coded by 16 polymorphic genes that are divided into five classes:  $\alpha$  (GSTA),  $\pi$  (GSTP),  $\mu$  (GSTM),  $\theta$  (GSTT),  $\zeta$  (GSTZ).<sup>75</sup>

GSTM1 and GSTT1 loci are very polymorphic and the deletion of both alleles (null genotype) eliminates the role of the enzyme and enhances the vulnerability towards oxidative stress.<sup>74</sup> It was reported that GSTT1 and GSTM1 polymorphisms affect the degree of cardiac siderosis as one of the factors that causes death in Thalassemia patients.<sup>76</sup> A "Null" genotype of the GST genes can lead to an enhancement of susceptibility to oxidative reactions, with the result of increased risk of tumor, prostates or cardiac disorders.<sup>77</sup> As well as CAT (coding gene of Catalase enzyme) polymorphism in exon 9 on the rs769217 locus is allegedly associated with low activity of Catalase enzyme which result in lower protection of catalase against free radicals.<sup>78</sup>

Osteopenia and osteoporosis are some of key factors in the morbidity of Thalassemia Major (TM) patients.<sup>79</sup> There are some factors leading to a reduction of bone density in TM, one of them could be mutations in gene for I collagen type, *COL1A1*.<sup>80</sup> I collagen type is the most abundant protein in bone matrix. Polymorphism of the gene is associated with bone mineral density (BMD). Mutation in SP1 binding site (G>T mutation) is reported as having a strong association with osteoporosis in TM patients. A study revealed that 19% of  $\beta$  thalassemia patients have a homozygous (G/G) genotype, 40% are heterozygous (T/G) and 43% are homozygous (T/T) genotype.<sup>81</sup>

Hyperbilirubinemia and a propensity to the gallstones formation are general complications of  $\beta$  thalassemia patients. The complication is caused by ineffective erythropoiesis and shortening of the erythrocyte age. TA sequence polymorphism in the gene promoter of uridine diphosphate-glucuronosyltransferase 1A (UGT1A), where the product is involved in bilirubin glucuronidation inside the liver also plays a role in the formation of gallstones.<sup>82</sup> In normal individuals, the UGT1A gene promoter has six replications of TA (TA 6). Individuals with seven replications (TA 7) tend to have high level of bilirubin. Thalassemia patients with this polymorphism have a high propensity to form a gallstone.<sup>83</sup>

$\beta$  Thalassemia patients, especially major and intermedia can develop complications in adult state such as hypercoagulable states, including deep venous thrombosis and pulmonary embolism as well as arterial thrombosis and stroke.<sup>84</sup> Study also reported that Thromboembolic events (TEE) were frequently developed in splenectomized patients.<sup>85</sup> Genetics features involved in TEE should be considered such as Factor V Leiden (FVL) rs6025, Prothrombin

G20210A rs1799963, PAI-1 -675 4G/5G, and MTHFR mutations.<sup>86</sup>

The interaction with the gene mutation that causes a deficiency of pyrimidine-5 nucleotides-I (P5N-I) can cause a severe hemolytic anemia due to the susceptibility of the individual to free radicals.<sup>87</sup> In addition, clinical complications of  $\beta$  thalassemia patients is also affected by a set of genes related to iron metabolism such as Human hemochromatosis (HFE), transferrin receptor 2 (TFR2), ferroportin (FPN), Hpcidin (HAMP) and Hemojuvelin (HJV) genes.<sup>88</sup>

## CONCLUSION

The studies of the genetic modifiers in thalassemia have been growing very rapidly. Some of the latest findings have good impact in clinical practices. The research on genetic of  $\beta$  thalassemia was needed for improvement in management patient in the future. MGP investigation should be done in high risk couples to predict whether the newborn will have mild or severe clinical manifestation.

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