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The Genetic Aspect of Thalassemia: From Diagnosis to Treatment

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<http://dx.doi.org/10.5772/intechopen.76496>

Abstract

Hemoglobinopathies are a group of red blood cell productivity disorders, including α -thalassemia, β -thalassemia, and sickle cell disease (SCD), that are autosomal recessive and very common in Mediterranean, Middle Eastern, East Asian, and African countries. Thalassemia presents with the following clinical signs and symptoms: fatigue, weakness, yellowish skin, facial bone deformities, and abdominal swelling. Genetic studies have successfully characterized the key variants and pathways involved in hemoglobin F (HbF) regulation, providing new therapeutic targets for HbF reactivation. According to the current literature, using lentivirus vector for gene therapy and genome-editing-based treatment strategies for β -thalassemia and SCD have been discussed and well documented. According to current studies, novel treatments are becoming more important for thalassemia patients, because the consequences of supportive treatments are not sufficient for patients and their families. Supportive treatment does not have a positive effect on the survival rate of β -thalassemia patients. New treatments are empowering to develop a gene therapy for β -thalassemia and include pharmacological or disruption of BCL11A erythroid enhancer by CRISPR-CAS9 technology in addition to zinc-finger or transcription activator-like effector nuclease, and attempts at repairing the defective β -globin gene in hematopoietic stem cells by genome editing. These approaches are needed to improve for being more successful; gene addition has the advantage of making use of a single product applicable to all cases of β -thalassemia.

Keywords: α -thalassemia, β -thalassemia, SCD (sickle cell disease), HSC (hematopoietic stem cell), lentiviral and CRISPR-CAS9 technology, HSPCs

1. Introduction

β -Thalassemia is the most common inherited hemoglobinopathy in the world and is on the increase in Mediterranean, Middle Eastern, and Asian populations. β -Thalassemia is a common

genetic disease caused by the coinheritance of two mutant β -globin alleles [1]. The β -thalassemias are inherited in an autosomal recessive disease. After fertilization, each patient has a 25% chance of being affected, a 50% chance of being an asymptomatic carrier, and a 25% chance of being unaffected and not a carrier. Heterozygotes (carriers) may have moderate phenotype and anemia but they clinically have no symptoms. Three clinical and hematological forms of increasing severity are recognized: β -thalassemia carrier, β -thalassemia intermedia, and β -thalassemia major (B-TM). The β -thalassemia carrier form, which results from heterozygosity for β -thalassemia, is clinically nonasymptomatic and is described by specific hematological findings. B-TM is a mild transfusion-dependent anemia. Thalassemia intermedia is known in terms of a clinical and genotypical heterogeneous group of diseases, varying in severity from the asymptomatic carrier to B-TM. The β -globin (HBB) gene maps in the short arm of chromosome 11, in a region including the δ -globin gene, the embryonic ϵ -gene, the fetal A-gamma and G-gamma genes, and a pseudogene. β -Thalassemia in the group of thalassemia syndromes is molecularly heterogeneous. Especially, genetic testing can provide important information for diagnosis, treatment, and prevention. There are some limitations to genetic testing in all types of thalassemia; the most important issue is to find the right patient on which to perform genetic testing [2].

Clinical management of B-TM involves routine long life red blood cell transfusions and iron chelation therapy to remove iron introduced in excess with transfusions. β -Thalassemia therapy modalities include curative allogeneic bone marrow or stem cell transplantation and symptomatic iron chelation therapy such as using deferoxamine. Therapies under investigation are the induction of hemoglobin F (HbF) with pharmacologic compounds and stem cell gene therapy [3]. The novel therapies are based on clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR-Cas9) system therapy and stem cell gene therapies.

The β -like globin genes are each expressed at distinct stages of development through a process referred to as Hb switching (embryonic \rightarrow fetal \rightarrow adult). At 6 months after birth, -HbF ($\alpha_2\gamma_2$), which comprises <5% of the total Hb, continues to fall reaching the adult level of <1% at 2 years of age, when adult Hb becomes the major Hb, and mutations affect the adult HBB gene. The HBB locus is a paradigm for tissue- and developmental stage-specific regulation; expression of the individual globin genes relies on a timely and direct physical interaction between the globin promoters and the β locus control region (β -LCR), the interaction being mediated through binding of erythroid-specific and ubiquitous transcription factors. The double mechanism has been suggested for developmental expression: (1) gene competition for the upstream β -LCR, presenting an advantage for the gene closest to the LCR [4], and (2) transcriptional suppression of the preceding gene. The ability to take part in β -LCR and transcriptional repression depends on the change in the abundance and list of different transcription factors that favor promoter-LCR interaction. While the ϵ - and γ -globin genes are autonomously silenced at the proper developmental stage, expression of the adult β -globin gene depends on the absence of competition from the upstream γ -gene for the LCR sequences. According to integrating molecular studies (such as mutational and epigenetic analyses), researchers have characterized a number of cis-regulatory genomic regions in the β -globin locus with a potential role in Hb switching. Naturally occurring deletions encompassing β - and γ -globin genes in the β -globin gene cluster and point mutations in the promoters of global

genes result in increased HbF expression and a benign situation called hereditary persistence of fetal Hb (HPFH). HPFH point mutations may disrupt binding sites for γ -globin silencers or generate new binding sites for γ -globin activators [5].

β -Thalassemia is characterized by reduced synthesis of the Hb β -chain that results in microcytic hypochromic anemia, an abnormal peripheral blood smear with nucleated red blood cells and diminished amounts of HbA on Hb analysis. Patients with T-BM have profound anemia and enlarged liver and spleen; they mostly come to medical therapy within the first 2 years of life [1]. β -Thalassemia is caused by a spectrum of mutations that results in a quantitative reduction of β -globin chains that are fundamentally normal. β -Globin is encoded by a structural gene found in a cluster with the other β -like genes on chromosome 11. The cluster includes five genes, δ (HBD), β (HBB), ϵ (HBE), $G\gamma$ (HBG2), and $A\gamma$ (HBG1), which are organized along the chromosome in order of their development expression to synthesize different Hb tetramers: embryonic Hb Gower-1, Hb Gower-2, Hb Portland, fetal Hb, and adult Hb. Expression of the globin genes is dependent on local promoter sequences as well as upstream β -globin LCR, which consists of five hypersensitive sites distributed between 6 and 20 kb 5' of HBE gene. All these regulatory regions bind a number of key erythroid-specific transcription factors, remarkably GATA-1, GATA-2, NF-E2, KLF-1, and SCL, as well as various cofactors and factors that are more ubiquitous in their tissue distribution, such as Sp1 [6].

Molecular genetic reports have been effective in describing the crucial variants and pathways involved in HbF regulation, furnishing novel therapeutic molecules for HbF reactivation. BCL11A has been well known as a quantitative repressor, and progress has been made in manipulating its expression using genomic and gene-editing strategies for therapeutic benefits. Studies and understanding in the mechanisms of ineffective and abnormal erythropoiesis have also provided further therapeutic molecules, two of which are now being tested in clinical experiments. BCL11A cooperates with SOX6, GATA1, FOG1, and NuRD complex to repress the expression of γ -globin genes in adult erythroblasts. The expression of BCL11A is regulated by KLF1 that favors fetal-to-adult Hb switching by directly activating β -globin gene expression. KLF1 is a key molecule involved in the γ - to β -globin Hb switching process by different mechanisms. In addition, KLF1 is a direct activator of genes that code repressors targeting recovery from the severity of β -thalassemia. The transcription factor lymphoma-related factor silences γ -globin expression through the NuRD complex. GATA-1 is the main gene regulating erythropoiesis and positively regulates specific erythroid genes such as erythropoietin receptor (EPoR), glycophorin (GpA), and globin chains, and is necessary for megakaryocyte and erythrocyte differentiation [7]. While the interaction of FOG1 with GATA-1 helps maintain erythroid homeostasis and control HBB transcription, KLF1 controls β -globin expression and switching between fetal and adult globin expression [8–11]. Several nuclear factors are believed to provide the Hb switching mechanism, including KLF1, MYB, the stage selector protein, and the nuclear receptors TR2/TR4 and COUP-TFII. The zinc-finger transcription factor BCL11A has a major role in the silencing of δ -globin expression in human cells. BCL11A is thought to exert this function by interacting with the erythroid master regulator GATA1, SOX6, FOG-1, and the NuRD complex (**Figure 1**). BCL11A is essential for the proper development of B cells, and murine BCL11A-deficient hematopoietic stem cells (HSCs) show defects in cell cycle, engraftment, and multilineage differentiation [11].

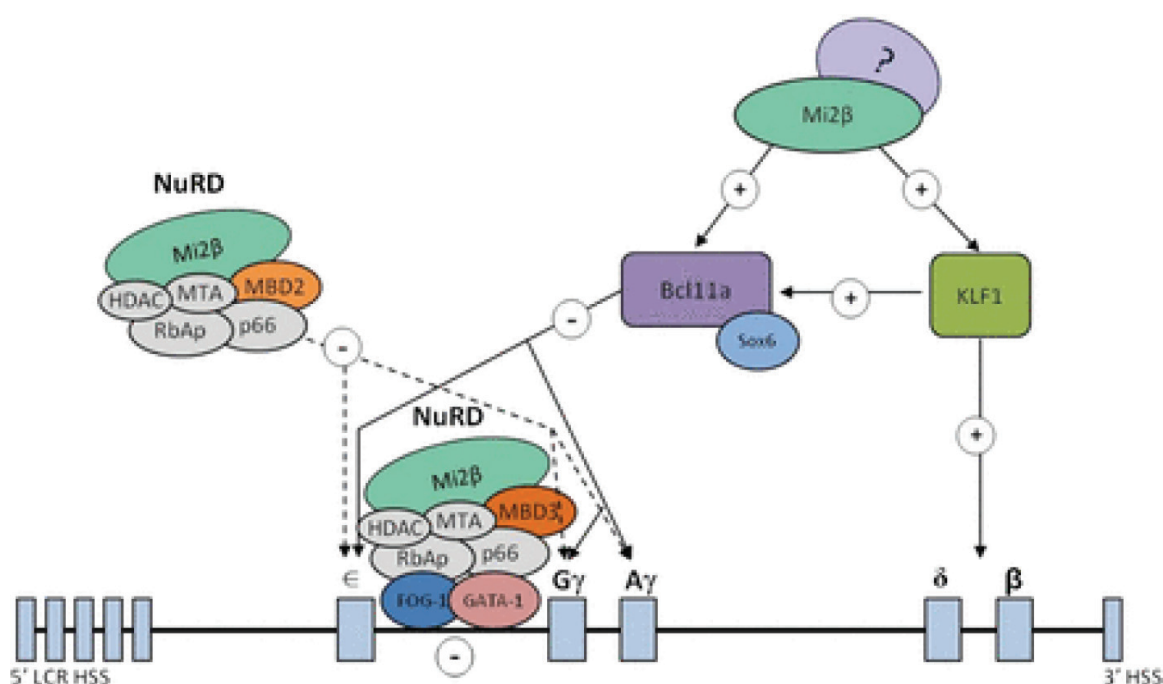


Figure 1. Major players in fetal-to-adult globin switching.

The manifestation of inherited mutations in KLF1 make HPFH suggest that this factor is a practical target for gene therapy and might be achieved by RNA interference technology to design a haploinsufficiency situation. Promising key molecules such as KLF1 and BCL11A for therapeutic efforts aimed at increased HbF level have been determined; however, further preclinical data are necessary before manipulation of transcription factors can be translated into therapeutic choices. According to some study groups, significant advances in the field by defining mechanisms through which BCL11A repressed γ -globin expression hold promise for the development of genome-based therapy in the future [12].

Hb is a tetramer comprised of both α - and β -like polypeptide subunits. According to ontogenesis, the constitution of these subunits varies, leading to assembly of Hb molecules with various physiologic assets. In humans and Old World monkeys, two developmental switches take place for the synthesis of the β -like subunits of the Hb molecule. The initial switch is present in all mammals and involves a switch from Hb subunits expressed entirely in the transiently produced embryonic primitive wave of erythrocytes to the Hb subunits produced in the earliest definitive wave of erythrocytes arising from the fetal liver. The Hb switch is well known as the primitive-to-definitive switch at the β -globin locus. It is important to note that at the α -globin locus in humans a similar switch from an embryonic Hb, which is normally restricted to primitive erythrocytes, to the adult α -globin subunits occurs [13].

2. Molecular diagnosis of β -thalassemia

Molecular diagnostic methods are mostly beneficial in giving genetic counseling and during the application of prenatal diagnosis. PCR-based tests are adequate for finding carriers of

α -thalassemia deletions, and applicable multiplex PCR and multiplex ligation-dependent probe amplification (MLPA) tests are suitable for diagnosing α - and β -thalassemia caused by deletions [14]. Diagnosis of β -thalassemia is more challenging, because of detecting the variety of diseases caused by mutations., Direct DNA sequencing might be the most practical

I. Promoter regulatory elements

- (1) CACCC box
- (2) ATA box
- (3) 5' UTR (CAP +1 to +45)

II. RNA processing splice junction

- (1) IVS1-position 1 and 2
- (2) IVS1-3' end (minor deletion of 17 to 44 bp and insertion of 22 bp)
- (3) IVS2-position 1 and 2
- (4) IVS2-3' end

Consensus splice sites

- (5) IVS1-position 5
- (6) IVS1-position 6
- (7) IVS1-positions -3, 128, 129
- (8) IVS2-5
- (9) IVS2-3' end cryptic splice sites
- (10) IVS1-positions 110, 116
- (11) IVS2-positions 654, 705, 745, 837
- (12) CD10 (GCC \rightarrow GCA)
- (13) CD19 (AAC \rightarrow AGC) Hb Malay (Asn \rightarrow Ser)
- (14) CD24 (GGT \rightarrow GGA)
- (15) CD26 (GAG \rightarrow AAG) (Glu \rightarrow Lys, HbE)
- (16) CD26 (GAG \rightarrow GCG) (Glu \rightarrow Ala, Hb Tripoli)
- (17) CD27 (GCC \rightarrow TCC) (Ala \rightarrow Ser, Knossos)++RNA cleavage—poly A signal.
- (18) AATAAA—single base substitutions, minor deletions; others—in 3' UTR.
- (19) Term CD +6, C \rightarrow G.
- (20) Term CD +90, del 13 bp
- (21) Term CD +47 (C \rightarrow G)

III. RNA translation initiation codon

- (1) ATG—single base substitutions, 45 insertion nonsense codons
 - (2) Numerous examples of single base substitutions, all leading to premature termination codons frameshift
 - (3) Numerous examples of minor insertions, deletions, shifting reading frame, and leading to premature termination codons
-

Table 1. Categories of the mutation in β -thalassemia [6].

method for detecting all mutations associated with β -thalassemia in the United States, due to having different ethnic populations. There is less heterogeneity in β -thalassemia mutations in populations in some parts of the world, and a more targeted strategy may be used to screen for β -thalassemia carriers in these areas of the world. Recently, reverse dot-blot hybridization, restriction fragment length polymorphism, and denaturing gradient gel electrophoresis have been used as the most commonly mutation screening methods. These tests are labor intensive with low throughput, need expensive high-technology equipment, and may give results that are difficult to interpret.

The most common mutation in β -globin gene is IVS-I-110 in Mediterranean countries, including Turkey, Italy, Egypt, and Greece. The Aldemir et al. study has shown the spectrum of β -thalassemia mutations in Hatay province, Turkey, and it established a foundation for prenatal genetic testing that will be a part of an effective prevention program for β -thalassemia disease. The spectrum of β -thalassemia mutations in 93 unrelated affected patients was determined by Aldemir et al. A large amount of β -thalassemia mutations was identified by them using a direct sequencing method. The results are different from the other parts of Turkey. The most common mutations were: IVS-I-110 (G > A), IVS-I-6 (T > C), IVS-I-1 (G > A), frameshift codon (FSC)8(–AA), codon 39 (C > T), and IVS-II-745 (C > G). They reported that many Syrian and Iraqi immigrants presented with a prevalence of thalassemia traits that were different from other studies [2]. β -Thalassemia is caused by more than 300 different β -globin chains (<http://www.ithanet.eu/db/ithagenes>; <http://globin.bx.psu.edu/hbvar>) but only about 40 account for 90% or more of β -thalassemia cases worldwide [6].

A wide spectrum of mutations interferes with the processing of the primary mRNA transcript. Those that affect the invariant dinucleotide GT or AG sequences at intervening sequence (IVS) prevent normal splicing altogether, causing β^0 -thalassemia. Both exons and introns also contain “cryptic” splice sites, which are sequences very similar to the consensus sequence for a splice site but are not normally used. Mutations can occur in these sites producing a sequence that looks like the normal splice site; however, it is not normally used. During RNA processing the created site is used differently, leading to unusual splicing; inadequately spliced mRNA is not functional because a frameshift and a premature termination codon are created by spliced intronic sequences. Such mutations in codon 26 in exon1 of HBB gene result in the HbE variant. Clinical manifestation of compound HbE/ β -thalassemia heterozygotes looks like those with two β -thalassemia alleles, extending between severe anemia and transfusion dependency and nontransfusion-dependent states (i.e. thalassemia intermedia or nontransfusion-dependent thalassemia (NTDT)), depending on the non-HbE/ β -thalassemia allele and other genetic factors. Other RNA processing mutants affect the polyadenylation signal and the 3' UTR. These are mild β -thalassemia alleles [5]. Those mutations are categorized in three different regulatory sites (shown in **Table 1**) [6].

3. Genetic factors on clinical severity, classical therapy, and novel treatments: gene therapy for β -thalassemia

The clinical severity of the β -thalassemia syndromes relies on the extent of α -globin chain/non- α -globin chain imbalance. The erythroid precursors in the bone marrow and in the spleen are

damaged by the α -globin chain, causing ineffective erythropoiesis. However, bone marrow examination is usually not needed for diagnosis of patients. The bone marrow is very cellular, mainly as a result of marked erythroid hyperplasia, with a myeloid/erythroid ratio reversed from the normal (3 or 4) to 0.1 or less. Clinical presentation of B-TM occurs between the ages of 6 and 24 months. The usual presentation is progressive pallor in the second half of the year with abdomen distention due to hepatosplenomegaly. After age 10–11 years patients are at risk of developing severe complications related to iron overload, depending on their obedience with chelation therapy [3].

Heterogeneity in the clinical expression of β -thalassemia diseases may occur from the nature of β -globin gene mutations, α -thalassemia gene interaction, or differences in HbF production. This study was undertaken to define whether these genetic determinant factors can predict the phenotypic severity of patients with β -thalassemia and to evaluate the relationship between the phenotype and genotype. According to the current literature, coinheritance of types of β -thalassemia mutation and α -thalassemia in a patient who has at least one allele of the genotype of β -thalassemia is predictive of the clinical severity of the disorder. Conversely, a mild clinical finding in some patients with β_o/β^+ -thalassemia or β_o -thalassemia /HbE is that these individuals do not have an α -thalassemia haplotype.

The β -thalassemia phenotype was found to be modified by different factors. β -Thalassemia with coinheritance of α -globin gene deletion may have moderate clinical features while deletion of both α -globin genes is characteristically related to thalassemia intermedia. HbF is the major modifier of disease severity in individuals with β -thalassemia. Because the severity of homozygous β -thalassemia is associated with the disproportion between α - and β -globin chains, even the low levels of γ -globin in F cells decrease the relative excess of α -globin and provide a selective survival of cells producing HbF at the time of ineffective erythropoiesis in the most common forms of β -thalassemia. Any factors that can decrease the degree of disproportion may develop the clinical feature. Thus, this selective survival might account for high levels of HbF in homozygous β -thalassemia. The presence of genetic variants continues to produce HbF, hence it has a strong influence on the clinical feature of development [12]. Higher levels of expression of HbF in adulthood have been suggested to improve the morbidity and mortality in sickle cell disease (SCD). Nemati et al. have studied the incidence of Xmn1 polymorphic sites in B-TM patients from western Iran. The study detected that having this polymorphic site caused a positive effect on HbF production and the G- γ ratio, which could improve the clinical findings of β -thalassemia patients [15].

4. Classical treatments in β -thalassemia

Classical treatments are classified into five subgroups. We discuss the benefits of these therapies and their long-term side effects below.

4.1. Transfusion treatment

Increasing evidence shows the advantage of transfusion therapy in decreasing the occurrence of complications such as pulmonary hypertension and thromboembolic events. Thus, although

the common practice was to start transfusion when complications arose, it may be valuable to initiate transfusion therapy earlier as preventive strategies arise, which will help ease the increased risk of alloimmunization with delayed start of transfusion. Although earlier introduction of blood transfusions will increase the ratio of iron accumulation, current approaches of iron chelation are available now. Transfusion becomes necessary when the sense of well-being of the patient diminishes to a level insufficient to enjoy the activities of a normal life. Problems such as chronic hypoxia for levels of Hb below 70 g/L usually develop in patients. The patient's general situation such as regular growth, growth velocity, size of the spleen, bone age, periods of rapid growth, bone deformities, and pregnancy must be considered. Transfusion sometimes becomes necessary during infection-induced aplastic crises. Heart disease is also an indication to transfusion therapy. The transfusion treatment should be similar to the one generally adopted for B-TM, when the decision to transfuse is made [4, 16, 17].

4.2. Splenectomy

The present indications for splenectomy in Thalassemia Intermedia (TI) involve growth retardation, increased transfusion requirement, leukopenia, thrombocytopenia, and splenomegaly [16]. Splenectomy, however, can contribute to a rising predisposition to thromboembolic events and pulmonary hypertension in TI. The presence of a chronic hypercoagulable situation could be due to the procoagulant influence of the anionic phospholipids exposed on the surface of the impaired circulating red blood cells [4]. Devastating postsplenectomy sepsis is a sudden event, and it can be mortal. The most frequent bacteria in postsplenectomy patients are *Streptococcus pneumoniae*, *Hemophilus influenzae*, and *Neisseria meningitidis* [18]. Iron-loaded macrophages lose the ability to kill intracellular pathogens by the interferon- γ -mediated pathways. The loss of this ability is associated with reduced formation of nitric oxide in the presence of iron. Splenectomy generally stops transfusion in the majority of patients. However, it does not generally modify the high output state and the increased pulmonary artery pressure that often characterizes thalassemia intermedia. Partial dearterialization of the spleen and partial splenectomy have a crucial and beneficial effect, but are not long-lived [19].

4.3. Iron chelation therapy

After one decade, iron chelation therapy was begun for preventing iron accumulation in some organs, but iron chelation may not prevent the development of clinical cardiac disease. A direct calculation of liver iron concentration is suggested, either by biopsy or by a noninvasive method such as R2 MRI. Iron chelation therapy should generally be started if liver iron concentration exceeds 7 mg/g dry weight of liver tissue. Threshold serum ferritin values of 400–500 ng/mL could be considered as an indicator for starting iron chelation therapy [19, 20].

4.4. Alteration of fetal hemoglobin production

Drugs increase levels of HbF ($\alpha_2\gamma_2$) and could be an advantage in patients with β -thalassemia intermedia because of an improvement in the balance of globin synthesis. Hydroxyurea is accomplished by inducing HbF synthesis and increasing γ -globin production. Both hydroxyurea

and butyrate by-products have shown only modest increases in Hb. Some patients with β -thalassemia intermedia, who are not transfusion dependent, may respond to hydroxyurea treatment. Hydroxycarbamide has been administered to thalassemia patients according to many different regimens, alone or in combination with other drugs. A significant decrease in the need for blood transfusions was observed in many patients; the need was completely removed in some patients. α -Deletions, the XmnI polymorphism, and HbE/ β -thalassemia may be predictive of a good response to hydroxycarbamide. The sense of well-being, almost generally reported, may imitate the significant decrease in ineffective erythropoiesis. Using recombinant human erythropoietin (rHuEPO) in some clinical trials for the treatment of thalassemia showed a significant, dose-dependent increase in thalassemic erythropoiesis, without an increase in HbF, mean corpuscle volume (MCV), and mean Hb content (MHC), and without an alteration in the α /non- α ratio, mostly in splenectomized patients with thalassemia intermedia. Combination therapy with erythropoietin and hydroxyurea in thalassemia patients appears to be more advantageous than either therapy alone with respect to HbF increase and an increased packed cell volume [19].

4.5. Antioxidants

Oxidative damage may be generated by the presence on the cell-free globin chain and labile plasma iron (LPI), a chelatable component of nontransferrin-bound iron, which can be reduced by treatment with iron chelating agents. The persistent stress on neutrophils can decrease their antibacterial ability and their respiratory eruption reaction. Combined administration of vitamin E with *N*-acetylcysteine and iron chelators could be more efficient than the use of a unique antioxidant [19].

B-TM patients' prognoses have considerably developed over the last decade with the advent of noninvasive methods to measure organ iron before the occurrence of clinical symptoms, new chelators, and increased blood safety procedures. The novel improvements in medicine have opened the way to an important development in diminishing cardiac mortality, previously reported to cause 71% of deaths in B-TM patients [5]. Studies show that despite ethnic differences, most individuals with transfusion-dependent thalassemia have normal cardiac iron but a significant proportion have simultaneous liver iron overload [21]. The novel and the most promising method for this purpose are gene-editing tools (GETs), which allow for site-specific genome editing and targeted transgene integration in an efficient and accurate situation. Transcription activator-like nucleases (TALEN), zinc-finger nucleases, and CRISPR/Cas9 cooperate with the major GETs. The nuclease systems can be approximately classified into two groups based on their type of DNA recognition: ZFN, TALEN, and meganucleases succeed specific DNA binding via protein-DNA interactions, Cas9 is targeted to specific DNA sequences by a short RNA key molecule that base-pairs directly with the target DNA, and protein-DNA relations have a role in its targeting. CRISPR/Cas9 is a simple and efficient GET for in vitro and in vivo systems. In recent decades, a great amount of research has been conducted for the management of β -thalassemia. One of the novel therapeutic strategies, β -globin gene targeting, is then differentiated into HSCs and returned to the patient. When using induced pluripotent stem cells (iPSCs), the most urgent topics to be considered are the

elimination of transcription factors not needed after induction and reestablishment of correct reprogramming in a way that the iPSCs are not developing into tumors [8, 22, 10].

Gene therapy strategies for β -thalassemia are so versatile; the common goal in these strategies is corrected β -globin gene defects in iPSCs and CD34⁺ hematopoietic stem pluripotent cells (HSPCs). There are two alternative ways to use these treatments: the first one is to use CD34⁺ HSCs and subpopulations may be corrected by gene therapy. The second one is to isolate and reprogram somatic cells (shown in **Figure 2**) [23]. The emergence of gene-editing technology, which enables precise genome application, offers a new strategy for treating β -hemoglobinopathies. Site-specific double strand breaks (DSB) can be induced with zinc-finger nucleases, TALENS, meganucleases, and more recently with the CRISPR/Cas9 system. CRISPR/Cas9 has revolutionized gene targeting. Unlike other nucleases, which use a protein dimer for target sequence recognition and require a novel protein to be engineered for each new target site, CRISPR/Cas9 technology uses a short guide RNA (gRNA) with a 20 bp sequence complementary to the DNA sequence to be targeted [24].

Gene therapy by either gene insertion or editing is a curative therapeutic option for hereditary blood cell disorders such as SCD and β -thalassemia. The safety and efficacy of gene transfer techniques has improved by using lentiviral vectors. This technology has developed perfectly, although there are some limitations, including number of engraftment-transduced HSCs and

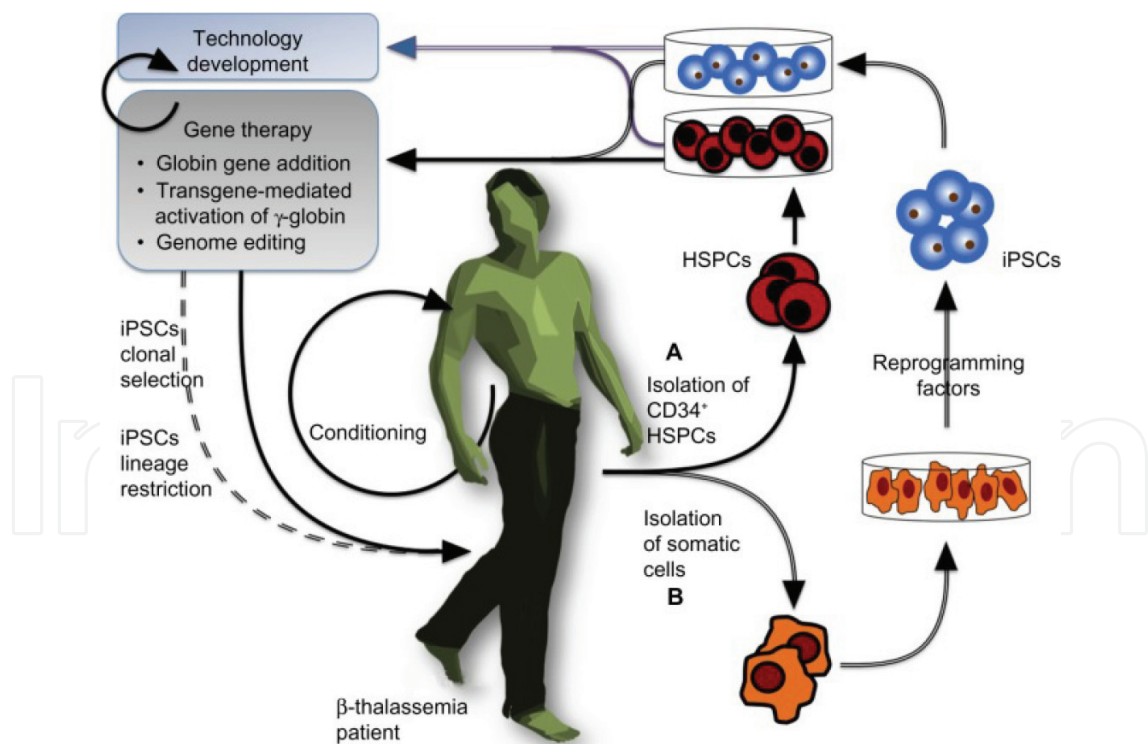


Figure 2. Gene therapy strategies for beta thalassemia. (A) The commonly used CD34⁺ HSPCs and subpopulations may be corrected directly by gene therapy. (B) Alternatively, somatic cells can be isolated and reprogrammed to pluripotency, with the resulting iPSCs then being a patient-specific substrate for gene therapy, clonal selection, and lineage-specific differentiation. Excepting circular arrows, solid arrows indicate procedures for HSPCs and hollow arrows those for iPSCs [23].

sufficient transgene expression that results in complete correction of β^0 -TM. This underlines the need to classify and address factors that might be contributing to the *in vivo* survival of the transduced HSCs or find means to develop expression from current vectors. According to recent knowledge, specific gene therapy methods for hemoglobinopathies are needed to be reviewed in the light of the success rate of preclinical studies and clinical trials. The preclinical trials will be needed to correct the side effect of viral vectors such as HIV-1 (LV), and murine Moloney leukemia virus (RV). The main problems are removing the genetic elements responsible for their virulence and pathogenicity, and adding the β -globin gene and its LCR. LV has been most useful at correcting hemoglobinopathy animal models and has resulted in their clinical translation. Initial studies looking at RV-mediated human β -globin gene transfer without inclusion of the LCR showed variable and low levels of gene expression. Following a number of studies, nearly 10 years of effort to develop RV for expressing effective globin gene expression was found to be inadequate. RVs utilizing the enhancer/promoter sequences of LTR to drive transgene expression of genes other than globin genes were the first ones to be used in clinical trials. Despite their clinical success in immunodeficiencies of patients' gene therapy, concerns about their safety emerged following reports of vector-mediated insertional mutagenesis. The RV vector insertions give rise to immortalization of primary hematopoietic progenitor cells. While the RV-LTR is a strong enhancer and upregulates transgene expression to very high levels compared to relatively weaker enhancers from the HIV-LTR and cytomegalovirus, it concurrently activates cellular protooncogenes flanking insertion sites. Moreover, methylation of the LTR can cause inactivation of the integrated transgene promoter and prevent long-term transgene expression. The structure of a self-inactivating (SIN) vector design deletes the LTR promoter/enhancer and allows the transgene expression to be driven by internal cellular promoters, reducing LTR enhancer-mediated genotoxicity and its methylation-induced inactivation [25].

The preclinical LV-mediated human studies were shown to rescue mouse models of β -thalassemia intermedia and B-TM. Imren et al. showed correction of β -thalassemia mice using a vector carrying β^{T87Q} gene, where a point mutation in the β -globin gene confers it with antisickling properties. However, multiple copies were required for adequate correction of the mouse thalassemia phenotype [25]. Miccio et al. used an LV vector carrying the β -globin gene linked to a minimized LCR HS2/HS3. It showed that the thalassemia phenotype in th3/+ mice can be fully corrected by transduced hematopoietic cells with 30–50% frequency having an average vector copy number of 1. Moreover, it was shown that genetically corrected erythroblasts having an *in vivo* survival advantage were necessary to investigate the utility of reduced intensity transplant regimens for clinical gene therapy studies. According to Malik et al., gene therapy for hemoglobinopathies is a reality now, with several patients cured of their β^0/β^E -thalassemia or with significant enhancement from β^0/β^0 -thalassemia and one patient with SCD, while others are showing modest transgene expression [26].

The severity of thalassemia disease tries to limit the current curative capacity of gene transfer technology. The challenges to effective clinical translation in hemoglobinopathies cover the dose of engraftable-transduced HSCs, the intensity of the preconditioning transplant regimen, and expression of the transgene. *In vivo* selection models can provide expansion of the few genetically modified engrafted HSCs. Improving vector potency will increase gene expression.

Efforts to promote differentiation of iPSC technology to produce engraftable HSCs can expand the HSC source, and gene editing can circumvent the need for high transgene expressing LV and potential, insertional mutagenesis of LV.

New technologies that can restore the future of gene therapy are gene editing using CRISPR-Cas9 and development of HSCs from iPSCs with long life repopulating potential, although this may need much work in reality. With scientific improvements in stem cell biology and genetic manipulation, it is considered that a future is possible where a child prenatally diagnosed with hemoglobinopathy can have her/his genetically modified cord blood stem cells transfused even before the fetal-to-adult Hb switch. This therefore will prevent the presence of any disease manifestations [25]. Lin et al. worked on a study to raise fetal hemoglobin for the treatment of β -thalassemia and SCD. They used CRISPR-Cas9 to modify normal bone marrow HSCs and HSPCs to the deletional HPFH genotype. The erythroid cells derived from such modified HSPCs showed significantly higher γ -globin expression compared with the nondeletion-modified CD34⁺ HSPCs, iPSCs. They made use of this clinical invention and modified CD34⁺ HSPCs to have part of the β -globin locus removed and repaired the genome by nonhomology end joining to create a genotype mimicking HPFH that produced a high level of γ -globin expression when differentiated into erythroid cells. The study showed that the CRISPR-Cas9 system is a new potential approach to autologous transplantation therapy for the treatment of homozygous β -thalassemia and SCD [27]. The current gold standard procedure for β -thalassemia patients involves a combination of granulocyte-colony stimulating factor (G-CSF) and plerixafor, a bicyclam molecule that antagonizes the binding of stromal cell-derived factor-1 expressed by bone marrow stromal cells to the chemokine CXCR-4 located on the surface of HSPCs. G-CSF plus plerixafor has been shown to provide very high numbers of CD34⁺ cells by single apheresis in mobilized thalassemic patients, despite the currently reported highest enrichment in HSCs using plerixafor alone [18]. In addition, two or three bone marrow harvests are required to collect an adequate dose of HSPCs from SCD patients; this is mainly because the HSC harvest is negatively influenced by (1) the inflammatory SCD bone marrow microenvironment and (2) the formation of cell aggregates during the isolation of bone marrow mononuclear cells. In patients with SCD, the administration of G-CSF led to severe adverse events and is therefore contraindicated. They suggested that plerixafor is potentially a safer mobilizing drug for SCD patients (<http://www.clinicaltrials.gov> identifier NCT02212535) [18].

In 2012, a phase 1 clinical study in β -thalassemic patients in the United States used the TNS9.3.55 vector expressing the wild-type β -globin transgene and a reduced intensity busulfan-based conditioning method. The result of this method was limited, and the four treated patients did not show sufficient clinical positive effect. A second phase clinical study is planned using a TNS9.3.55 variant vector, patient disease-symptoms free, although results need to be reproduced in a large number of patients and over a longer follow-up time (**Table 2**). For β^0 -thalassemia and possibly SCD, further improvements in manufacturing, cell processing, and protocol design are needed for clear clinical benefit. Several groups have proposed alternative LV and genome-editing-based strategies for raising endogenous γ -globin expression or correcting the disease causing mutations. However, LV vectors are regularly and safely used to genetically modify patient HSCs, and the clinical application of genome-editing approaches must be confirmed in terms of editing efficiency of long-time HSCs for the possibility of delivering associated toxicity

Vectors trans gene	Erythroid enhancer	Key findings	Key problems and possible solutions
TNS9 β -globin	HS2-3-4	Correction of anemia in thalassemia intermedia mice and rescue of lethality in mouse model	Variable human β -globin expression in thalassemia.
Beta ⁸⁷ β ^{T87Q} -globin	HS2-3-4	Correction of anemia in thalassemia intermedia mice. High-level expression of antisickling globin in erythroid cells	Multiple copies are required for correction; gene expression surrounding the integration sites was not analyzed
D432 β γ -globin γ	HS2-HS3-HS4	Correction of anemia in thalassemia intermedia mice	Variable phenotypic improvement in thalassemia intermedia mice due to chromosomal position effects
LAR $\beta\Delta\gamma$ γ -globin V5	Extended HS2-HS3-HS4	Correction of anemia in thalassemia intermedia mice	Improved $\beta\gamma$ -globin expression and reduced position effects
BG-I β -globin	HS2-HS3-HS4	Correction of human thalassemia major phenotype in vitro and in immune deficient mice [28]. Reduced position effects and uniform expression Identification of minimal regions of cHS4 necessary for optimal insulation	Low viral titers with full-length cHS4 insulator. Identification of regions of cHS4 that impart optimal insulation and have minimal effect on vector titers
T10 β -globin	HS1-HS2-HS3-HS4	Correction of anemia in thalassemia intermedia mice with lower vector copies	Low viral titers with full-length cHS4 insulator. Identification of regions of cHS4 that impart optimal insulation and have minimal effect on vector titers
HS40-11 γ -globin	HS-40	Partial correction of mouse γ -thalassemia intermedia with high transduction levels	γ -Globin expression insufficient for correction of thalassemia major
GLOBE β -globin	HS2-HS3	Correction of β -thalassemia major by gene transfer in murine [29] and human thalassemia hematopoietic progenitors	High vector copies are required for correction of thalassemia major mouse model

Table 2. Comparisons of different β -or γ -globin vectors studied successfully in mouse and human models of β -thalassemia.

and the potential off-target activity of the specific genome-editing tools [18]. Current clinical studies discovering alternate strategies such as matched distinct donors and development of novel regimens using haploidentical donors will enhance the transplant option for the majority of SCD patients in the future [28]. The other strategy to cure β -thalassemia is efforts to develop gene therapy. Two β -thalassemia patients with an altered β -globin LV-based vector (gene therapy) had been treated successfully. This improvement holds potential for SCD [30–32].

β -Thalassemia is the second common hereditary hemoglobinopathy after SCD. The discovery of gene therapy for β -thalassemia has been right by HLA-identical donors, the narrow window of application of HSC transplantation to the youngest patients, and recent advances in HSC-based gene therapy. A large number of publications on this topic show that gene therapy has the potential to become the therapy of choice for patients who lack either an HLA genotypical sibling or an alternative, medically acceptable donor. Genetically modified HSCs are a good alternative to allogeneic hematopoietic stem cell transplantation (HSCT) for treating

β -hemoglobinopathies. It supersedes the need for a matched donor and thus avoids the risk of graft versus host disease and graft rejection after HSCT. Furthermore, the conditioning regimen required for the engraftment of genetically modified cells, because of their autologous origin, does not include immunosuppressive drugs. The worldwide application of this treatment may be possible nowadays, thanks to better safety and the absence of treatment-related mortality in gene therapy trials to date [18]. In terms of the current literature, using novel LV vectors for gene therapy and genome-editing-based treatment strategies for β -thalassemia and SCD is discussed and well documented. Novel therapeutic strategies are based on the use of LV vectors and/or genome-editing tools to reactivate endogenous HbF expression [18, 27].

Several approaches can be used to genetically manipulate HSCs and correct the genetic defect underlying β -hemoglobinopathies. A large number of current clinical trials (based on the transplantation of autologous β -thalassemia and SCD HSCs) are investigating the use of LV vectors that express β -like globin transgenes. Their results support further evaluation of integrase defective lentiviral vectors (IDLV) as a new HIV-1 vaccine delivery platform [18, 26, 29]. Clinical trials of globin-expressing LVs are now under way at several sites in Europe and the United States. The initial results are encouraging with regard to the accomplishment of transfusion independence. Despite these promising results, some issues deserve further investigation. The purpose of the conditioning method is to eliminate diseased HSCs effectively while avoiding untargeted toxicity in other organs [26]. Novel treatment strategies are based on the use of LV vectors and/or genome-editing tools to reactivate endogenous HbF expression.

Traxler et al. used the CRISPR/Cas9 system to regulate a 13 bp HPFH deletion in the γ -globin promoters via microhomology-mediated end joining. This region is thought to contain a binding site for HbF repressor BCL11A. In their study, HbF reactivation was associated with reduced levels of sickle β -globin and ameliorated the SCD cell phenotype in vitro. Recreating levels of HPFH requires the nonhomolog end joining (NHEJ)-based excision of long genomic fragments containing the β - and δ -globin genes and putative 3.5 kb D-gamma-intergenic HbF silencer targeted by BCL11A. This approach takes advantage of the NHEJ repair pathway, which might decrease the overall efficiency of genome editing. Nonetheless, if the proof of principle works, this approach reactivated HbF, constantly disrupted the β -globin gene, and ameliorated the SCD cell phenotype [18, 33, 34]. The prestige of potential clinical studies in gene therapy is upcoming and an open clinical study for β -thalassemia is included in **Table 2**. The reprogramming of somatic cells into induced pluripotent stem cells opens a new approach for treating β -thalassemia. Ye and coworkers reprogrammed the skin fibroblasts of a patient with homozygous β^0 -thalassemia into induced pluripotent stem cells, which produced Hb. These reports that induced pluripotent stem cells could offer a new approach for the treatment of β -thalassemia. These strategies are at an in vitro level at the present time [35]. Epigenetic therapy approaches for β -thalassemia are promising for the cure for this disease.

This chapter outlined the key molecules of the molecular mechanism underlying β -thalassemia in relation to the development of novel treatments and an update is given both at clinical and preclinical trials. Gene therapy has reached an important point and phase 1 clinical trials have been launched to discover the effectiveness and particularly long-term safety [36].

Finally, the efficiency of genome editing and potential side effects (toxicity) of this strategy need to be further tested in well-intentioned HSCs. The different strategies must be compared in terms

of efficiency, efficacy, and safety to provide patients suffering from β -hemoglobinopathies with the best therapeutic option. Recently, several research groups have discovered genome-editing-based methods for correcting β -thalassemia mutations [18]. Ultimately, innovative trials have developed an enthusiasm that fully differentiated somatic cells can be reprogrammed to make stimulated pluripotent stem cells [32]. Subsequent trials showed alteration of a mouse model of SCD using this original strategy, and hence have paved the way to using these cells to treat hemoglobinopathies with a few restrictions: one restriction was the inability to repair all hematopoietic lines with induced pluripotent stem cells, which prevents using human treatment [37–39]. The future of gene therapy has exhibited remarkable advances. Phase 1 trials are already recruiting individuals with the goal to examine the effectiveness and mostly the long life safety of transplantation of autologous CD34⁺ erythroid progenitor cells transduced ex vivo with LV β -globin vectors [36].

Acknowledgements

The author thanks Mr. Ahmet Yildirim for technical language support in the preparation of this chapter. There was no financial support for this study. The author is very thankful to the IntechOpen Editorial Board for this publication.

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