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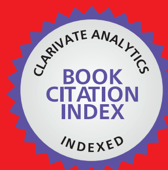

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Chapter 8

Novel Therapy Approaches in β-Thalassemia Syndromes — A Role of Genetic Modifiers

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61023

Abstract

The β-thalassemia syndromes are heterogeneous autosomal recessive hereditary disorders, caused by alterations in the *HBB* gene and characterized by absent or reduced β-globin chain synthesis. The β-thalassemia phenotypes are variable, ranging from severe, transfusion-dependent thalassemia major to mild, asymptomatic thalassemia trait. This interpatient clinical variability has swayed researchers toward identifying genetic modifiers for these disorders. Primary modifiers refer to type of alterations affecting β-globin gene. Secondary modifiers include variations in genes affecting α/β-globin chain equilibrium, such as genes involved in the γ-globin gene expression and genes affecting the amount and stability of α-globin chains. Tertiary modifiers are gene variations affecting the phenotype with regard to the complications caused by β-thalassemia syndromes. A role of secondary genetic modifiers in ameliorating the clinical phenotype has been observed. Secondary genetic modifiers are the most common targets for modern therapy and could be located within α- and γ-globin genes or outside globin gene cluster. The most potent secondary modifier genes are γ-globin genes. Production of fetal hemoglobin (HbF) trough adulthood ameliorates the severity of β-thalassemia phenotype. Large family and genome-wide association studies have shown that regions outside of the β-globin gene cluster are also implicated in γ-globin gene expression regulation. *HBS1-MYB* intragenic region and *BCL11A* gene have been particularly studied. Variants within these loci, along with γ-globin gene variants, account for approximately 50% of the HbF level variation, suggesting that additional factors are involved (transcription regulators (KLF1), regulators of α-globin chain stability (AHSP), epigenetic regulators (FoP)). Until recently a definitive cure for β-thalassemia could be achieved with bone marrow transplantation. However, it is available for less than 30% of the patients and bears a significant risk of morbidity and mortality. Alternative strategies, such as gene

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therapy and development of induced pluripotent stem cells (iPSCs) have been explored. The targets for gene therapy are hematopoietic stem cells, which are harvested from patient bone marrow or peripheral blood, purified by immunoselection, transduced by "therapeutic gene" aimed at correcting the effect of defective β-globin gene, and returned to the patient. Various types of vectors have been considered for gene transfer, including non viral (tRNK and ribozymes) and viral (retroviral and lentiviral vectors). In the past few years, iPSCs emerged as an interesting candidate for gene transfer. The feature that makes these cells appealing in the field of gene therapy is their susceptibility to gene correction by homologous recombination. Therapy protocols based on molecular basis of β-thalassemia are the best example of novel approaches in disease treatment.

Keywords: β-thalassemia, modifier genes, gene therapy, iPSC

1. Introduction

1.1. Hemoglobinopathies

Hemoglobinopathies are very heterogenic group of hereditary anemias and are classified according to the qualitative nature of the resulting hemoglobin and the quantitative amount of hemoglobin produced.

"Qualitative" hemoglobinopathies, or structural hemoglobin variants, are characterized by amino acid sequence variation of the hemoglobin (Hb) molecule which influences biochemical features of Hb. "Quantitative" hemoglobin disorders or thalassemias, are classified according to the deficient globin chain and, hence, include α-thalassemia and β-thalassemia.

Moreover, there is a distinct group of disorders classified as β -thalassemia syndromes which comprises β-thalassemia and certain group of hemoglobin variants whose synthesis is reduced due to amino acid sequence variation in the regions responsible for regulation of transcription or peptide stability.

Molecular mechanisms leading to formation of "quantitative" Hb variants include a δ-β hybrid gene (Hb Lepore) [1] and mutations causing hyperunstable beta globin chain synthesis (Hb Sabine) [2]. These highly unstable Hb variants precipitate before assembling with the α-globin chains to produce the Hb tetramer, resulting in excess of the α-globin chains and, thus, ineffective erythropoiesis [3, 4].

1.2. β-thalassemia syndromes

The β-thalassemia syndromes are one of the most common autosomal recessive hereditary disorders worldwide, with high prevalence in the populations of the Mediterranean, Middle-East, Central Asia, Indian subcontinent and Far East [3]. The β-thalassemias are caused by alterations in the β-globin gene and characterized by absent (β^0-thalassemia) or reduced (β^+-

thalassemia) synthesis of β-globin chain of adult hemoglobin (HbA; $\alpha_2\beta_2$). The result of this reduced globin chain synthesis is reduced production of functional hemoglobin tetramers which leads to hypochromia (decrease in the hemoglobin content of the erythrocytes) and microcytosis (reduces mean corpuscular volume of erythrocytes). Also, the excess of unbound α-globin chains precipitate in erythroid precursors in the bone marrow and red blood cells in circulation, leading to their premature death and, hence, to ineffective erythropoiesis and hemolytic anemia [5].

The clinical manifestations of β-thalassemias are extremely diverse, ranging form the severe, transfusion-dependent thalassemia major to the mild, asymptomatic thalassemia trait. Diverse phenotypes between the two extremes of thalassemia major and thalassemia trait constitute the clinical syndrome of thalassemia intermedia [6].

1.3. β-globin gene locus

The β-globin gene (*HBB*) maps in the short arm of chromosome 11, in the 70 kb region that also contains four other functional globin genes: embryonic, ε-globin gene (*HBE*), the fetal $^A\gamma$- and $^G\gamma$-globin genes (*HBG1* and *HBG2*) and adult δ-globin gene (*HBD*) as well as ψβ pseudogene. The five functional globin genes are arranged in the order of their developmental expression [7]. Upstream of the β globin complex is the locus control region (LCR), important regulatory region which consists of five erythroid-specific DNase1 hypersensitive (HS) sites (HS 1–5) distributed between 6 and 20 kb 5′ of the ε-globin gene (Fig.1). These HS sites interact with each other and two additional 5′ and one 3′ HSs to form an active chromatin hub (ACH) through looping, which interacts with the specific globin gene in any given moment of development. The role of the individual HS is still unclear, although HS2 and HS3 appear to be the most important sites for the efficiency of transcription, each leading to an ~30% loss of transcription when deleted [6, 8].

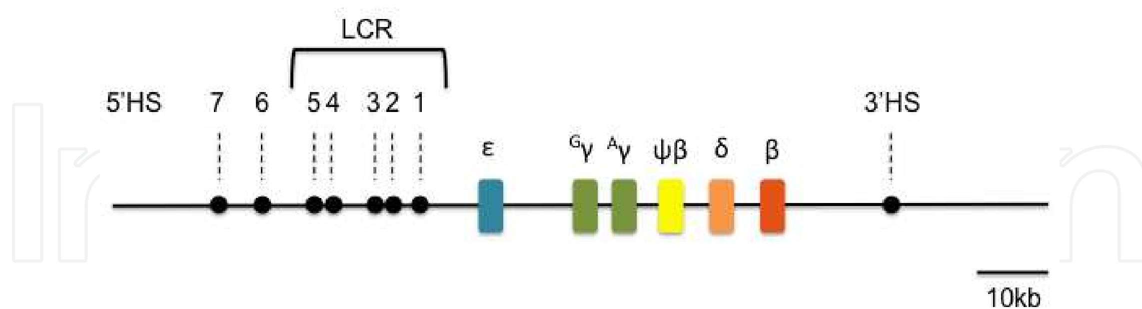

Figure 1. Structure of β-globin gene cluster. Human β-globin gene locus showing embryonic (ε), fetal ($^A\gamma$;$^G\gamma$) and adult (δ;β) globin genes, controlled by locus control region (LCR) and additional hypersensitive sites (3'HS; 5'HS). Region between fetal and adult globin gene contains ψβ pseudogene. The five functional globin genes are arranged in the order of their developmental expression.

During fetal development and the first six months of neonatal life, a complex pattern of globin gene expression occurs called the hemoglobin switch. Namely, in the early part of the first trimester, there is high expression of an embryonic globin gene within the primitive lineage of erythrocytes in the yolk sac. During fetal development, however, predominantly expressed

globin genes are γ-globin genes, coding for the γ-globin polypeptides, produced within fetal liver. These γ-globin chains combine with adult α-globin chains into a stable tetramer forming fetal hemoglobin (HbF; $\alpha_2\gamma_2$). Shortly after the time of birth this fetal hemoglobin is progressively replaced by the adult hemoglobin (HbA), which is mediated by a transcriptional switch in definitive erythroid progenitors from γ- to β-globin [9, 10].

2. Genetic modifiers of β-thalassemia

The β-thalassemia is caused by more than 200 point mutations and, rarely, by deletions [11]. However, genotypic variability at known loci is often insufficient to explain the phenotypic variability between individuals with the same genotype [12]. This interpatient clinical variability in the β-thalassemia syndromes has swayed researchers toward identifying genetic modifiers of severity for these disorders. Such genetic modifiers could potentially lead to the development of more specific and effective therapies [13]. Genetic modifiers exert their potential on three levels: primary, secondary and tertiary.

Primary modifiers usually refer to a type of alterations affecting β-globin gene. Location of the mutations within different gene regions determines the phenotypic severity, therefore the point mutations affecting the β-globin expression belong to three different categories: mutations leading to defective β-globin gene transcription (promoter and 5′ UTR mutations); mutations affecting mRNA processing (splice-junction and consensus sequence mutations, polyadenylation, and other 3′ UTR mutations); and mutations resulting in abnormal mRNA translation (nonsense, frameshift, and initiation codon mutations) [3]. Mutations affecting transcription usually result in a mild deficit of β-globin production that reflects the relatively mild phenotype of these β^+-thalassemias. The example of transcription affecting mutation is the C>T mutation at position -101 to the β-globin gene which appears to cause an extremely mild deficit of β-globin, such that it is asymptomatic in heterozygotes who have normal HbA_2 ($\alpha_2\delta_2$) levels [6]. Mutations affecting β-globin mRNA processing are located within 5′- and 3′-splice junction (donor and acceptor site), as well as within splice junctions' consensus sequences. Mutations altering the donor and acceptor splice site lead to deficiency of functional mRNA production resulting in complete absence of β-globin polypeptide chains and, hence, to β^0-thalassemia. On the other hand, mutations affecting consensus sequences surrounding splice-junction, decrease the efficiency of the normal splicing to varying degrees, hence producing β-thalassemia phenotype that ranges from mild to severe [7]. Also, these mutations could affect cryptic splice site, sequence that mimics a consensus sequence, leading to low efficiency splicing and therefore milder form of β-thalassemia. Cap-site mutations, as well as mutations affecting polyadenylation also lead to mild, β^+-thalassemia phenotype [14]. Mutations disrupting the mRNA translation ether in initiation or elongation phase, result in β^0-thalassemia phenotype. Most of these defects result from the introduction of premature termination codons due to frameshift or nonsense mutations and nearly all terminate within first and second exon [15].

Secondary modifiers include variations in genes affecting α/β globin chain equilibrium such as α- and γ-globin genes, as well as genes involved in the γ-globin gene expression (*HBS1-*

MYB, *BCL11A*, *KLF1, C1orf77*) and genes affecting the amount and stability of α-globin chains (*AHSP*). These genetic modifiers could be located within (α- and γ-globin genes) or outside globin gene cluster. In the recent years, there has been significant advancement in the fields of secondary genetic modifiers ameliorating the clinical phenotype of β-thalassemia syndromes. Specifically, production of fetal hemoglobin (HbF) trough adulthood could ameliorate the severity of β-thalassemia phenotype since γ-globin polypeptide chains compensate for the lack of the functional β-globin polypeptide chains. This is why γ-globin genes, along with other secondary modifiers, represent the most common targets for modern therapeutic.

Tertiary modifiers are gene variations affecting the phenotype with regard to some of the complications caused by β-thalassemia syndromes such as hyperbilirubinemia, propensity to gallstone formation, bone diseases, thrombophilia and cardiopathies [6].

Hyperbilirubinemia and gallstone formation (cholelithiasis) is a common complication of β-thalassemia and is attributed to the rapid turnover of the red blood cells, bilirubin being a breakdown product of hemoglobin. It occurs with variable incidence in homozygous β-thalassemia with the reported variation being partly related to the age of the patients and to its clinical severity, as it is more common in thalassemia intermedia than thalassemia major. Studies have shown that the levels of bilirubin and the predisposition to gallstones in β-thalassemia is related to a polymorphic variant in the promoter of the uridine diphosphate-glucoronyltransferase A1 (*UGT1A1*) gene, also referred to as Gilbert's syndrome. Individuals who are homozygous for the this (TA)7 variant instead of the usual (TA)6, tend to have higher levels of bilirubin and increased predisposition to gallstone [16, 17].

Progressive osteoporosis and osteopenia is another increasingly common complication observed in young adults with β-thalassemia and it is determined by a combination of genetic and environmental factors. Anemia and bone marrow expansion which are prevalent in β-thalassemia are major contributors in inadequately treated patients. Bone mass, the main indicator of the osteoporosis and osteopenia, is another quantitative trait known to be under strong genetic control involving multiple loci including estrogen receptor gene, vitamin D receptor (*VDR*), collagen type a1 and type a2 genes (*COL1A1*, *COL1A2*), and transforming growth factor β1 (*TGFβ1*) [15].

The presence of a higher than normal incidence of thromboembolic events, mainly in β-thalassemia intermedia patients, and the existence of prothrombotic hemostatic anomalies in the majority of the patients, have led to the recognition of the existence of a chronic hyper-coagulable state in thalassemic patients [18]. Genetic risk factors for thrombosis, such as mutations in the gene for Factor II and Factor V, as well as variations in the *MTHFR* gene, could significantly influence the phenotype of the β-thalassemia syndromes [19].

Cardiac diseases are the main cause of death in β-thalassemia patients and are attributed to iron overload because of regular transfusions, increased iron intestinal absorption, and ineffective erythropoiesis during the life span of the patients. One of the studies depicted apolipoprotein E4 and it decreased antioxidant activity, as a risk factor for left ventricular failure (LVF) in thalassemia patients. However, the presence of this E4 allele does not guarantee

the development of LVF in β-thalassemia but, when present, does affect the severity of the disease [20, 21].

3. Secondary genetic modifiers of β-thalassemia

3.1. Genetic modifiers of β-thalassemia within globin gene loci

3.1.1. α-globin genes

The α-globin genes encoding the α-globin chains are duplicated (α_1- and α_2-globin genes) and localized in the telomeric region of chromosome 16 (16p13.3), in a cluster containing also an embryonic, ζ-gene, encoding the embryonic globin chains, three pseudogenes (pseudo ζ-, pseudo α_1- and pseudo α_2-gene) and θ-gene of unknown function [22]. The level of transcription of the two α-globin genes differs, as the α_2 gene encodes two to three times more α-globin than α_1 gene. The different expression of the two α-globin genes has implications for the amount of hemoglobin variant present in carriers of α_1- or α_2-globin mutations and for the pathophysiology of the deletional and nondeletional forms of α-thalassemia [23]. As with β-thalassemia, α-thalassemias are characterized by absent (α^0-thalassemia) or reduced (α^+-thalassemia) production of α-globin chains, thus resulting in globin chain imbalance. The majority of the α-thalassemia defects result from deletions involving one or both α-globin genes on the same chromosome whereas point mutations affecting the functional expression of one of the two α-globin genes are not as common [24].

In many populations in which β-thalassemia is prevalent, α-thalassemia occurs at high frequency as well, resulting in coinheritance of both conditions. In these cases, homozygotes or compound heterozygotes for β-thalassemia will exert less severe phenotype since they would have less redundant α-globin chains. Therefore, the degree of amelioration of β-thalassemia phenotype depends on the severity of the β-thalassemia alleles and the number of functional α-globin genes. At one extreme, homozygous β-thalassemia patients who have only one functional α-globin gene, have thalassemia intermedia. On the other hand, the presence of increased α-globin product, as a result of triplicated or quadruplicated α-globin genes in β-thalassemia heterozygotes, increase the globin chain imbalance, converting a typically clinically asymptomatic state to that of thalassemia intermedia [25]. However, the phenotype of a single extra α gene ($\alpha\alpha\alpha/\alpha\alpha$) with heterozygous β-thalassemia is more variable and depends on the severity of the β-thalassemia allele [26, 27].

3.1.2. γ -globin genes

As already discussed, after a brief period of embryonic globin gene expression, the γ-globin chain of fetal hemoglobin (HbF) is predominantly expressed for much of gestation. Shortly after the time of birth, γ-globin is progressively replaced by the β-globin chain of adult hemoglobin (HbA).This complex process of globin gene expression called hemoglobin switch, is clinically important because the persistence or reactivation of γ-globin gene expression could mitigate the severity of the symptoms caused by β-thalassemia syndromes [9, 10].

Hereditary persistence of fetal hemoglobin (HPFH) is a condition characterized by continued γ-globin gene expression and, therefore, synthesis of high levels of HbF (1.6-30%) during adult life without other hematological abnormalities in affected heterozygotes. Two major types of HPFH have been described. Very high levels of fetal hemoglobin synthesis and uniform distribution of HbF among all RBCs characterize pancellular HPFH. On the other hand, heterocellular HPFH results from inherited increase in the number of erythrocytes with persisting production of fetal hemoglobin, termed F cells [28, 29].

Pancellular HPFH could further be divided into two classes. The deletional HPFH is caused by large deletions 3′ to the γ-globin genes, removing δ- and β-globin genes and part of the γδ intergenic DNA. These deletions appear to bring enhancer sequences into the proximity of the remaining γ-globin genes or remove specific regulatory sequences that play role as silencing elements for γ-globin genes, which in both cases promote high expression of fetal globin genes [4, 30]. Nondeletional form of HPFH are usually the result of point mutations (including small deletions) 5′ from the *cap* site of γ-globin gene promoters [28]. These point mutations occur in transcription factor binding motifs in the γ-globin gene promoters, clustered in three regions, around positions -114 to -117, at -175, and from -195 to -202 nucleotide [31]. The first region contains distal CCAAT box, responsible for binding basal CP1 and CDP transcriptional factor and erythroid-specific GATA1 and NF-E3 transcriptional factors [32]. Region which contains mutation g.-175 T>C contains binding site for GATA1 transcriptional factor while mutations affecting -195 to -202 region alter the affinity for binding of the Sp1 transcriptional factor [33-35]. Recent studies on γ-globin gene promoter, showed the presence of mutations associated with HPFH, outside of the known, -202 to -110, region. Namely, mutation g.-567 C>G upstream of the γ^{G}-globin gene (*HBG2*) alters a GATA1 binding motif which acts as a silencer of *HBG2* and is, thus, associated with increased γ-globin gene expression in affected adults [36].

Heterocellular HPFH is usually a result of mutations outside the β-globin gene cluster, such as locus located in the X chromosome. Namely, it was shown that a locus controlling F-cell production (FCP locus) is localized on Xp22.2 [37]. Despite these original findings, Xp22.2 loci affecting HbF levels were not supported by later genome-wide association studies (GWAS) [38, 39]. In some cases, elevated levels of HbF are observed in otherwise normal individuals, while in others, high HbF levels become apparent only when erythroid stress-producing factors are present [4, 30].

One of the common genetic variants that has little effect in normal individuals but favors a higher HbF response in the conditions of erythroid stress is XmnI-γ^{G} (g.-158 C>T) variant. This may explain why the same mutations on different chromosomal backgrounds are associated with different clinical severity. However, the HbF response associated with the XmnI-γ^{G} site is usually moderate and may not be sufficient to explain the wide difference in phenotype observed in some cases [26, 40]. Also, numerous studies do not support this positive effect of XmnI-γ^{G}, as a sole determinant on γ-globin gene expression [41, 42], which makes this issue very controversial.

Recently, large family and genome-wide association studies have shown that regions outside of the β-globin gene cluster are also implicated in γ-globin gene expression and HbF regulation. Two of them have been particularly studied: *HBS1-MYB* intragenic region and *BCL11A* gene.

3.2. Genetic modifiers of β-thalassemia outside globin gene loci

3.2.1. HBS1L-MYB intragenic region

Variants within *HBS1L-MYB* intragenic region, located on chromosome 6q23, account for more than 20% of the HbF level variance in northern Europeans. Most of these variants are distributed within 79 kb long region, which consist of three linkage disequilibrium blocks, referred to as *HBS1L-MYB* intergenic polymorphism (HMIP) blocks 1, 2, and 3. A small number of the variants shown to display an especially strong association with the increased levels of HbF, are concentrated in 24 kb of HMIP block 2, located 33 kb upstream of *HBS1L* and 65 kb upstream of *MYB* and include polymorphisms rs28384513, rs9399137 and rs4895441 [43, 44]. Despite strong genetic evidence and extensive studies, a clear mechanism trough which these variants are causing variation in HbF levels has remained elusive, although the two flanking genes, *HBS1L* and *MYB* are candidate target genes. Namely, recent data suggest that this intragenic area contains GATA1 binding motifs and is thought to have properties of regulatory element [44].

HBS1L gene is thought to be housekeeping gene because it is ubiquitously expressed. The function of *HBS1L*, a possible member of the "GTPases" superfamily, in the red blood cell development, and therefore in the regulation of HbF levels, is not immediately apparent and could be manifested indirectly, through its effect on the expression of various cytokines and transcription factors that impact erythroid cell growth [38, 45]. On the other hand, *MYB* gene, encoding the c-MYB transcriptional factor, is a well known regulator of hematopoiesis and erythropoiesis. This transcriptional factor plays an essential role in controlling the erythroid cellular proliferation/differentiation and acts as a potent negative regulator of HbF expression, trough which other genes, such as miR-15a and miR-16-1, play a role in HbF level variations [46, 47].

Although 6q22–23 locus, containing *HBS1L-MYB* intragenic region, have been extensively studied for over a decade, not enough attention had been paid in regard to other positional candidate genes in this quantitative trait loci (QTL). These include phosphodiesterase 7 (*PDE7B*), mitogen-activated protein kinase kinase kinase 5 (*MAP3K5*), and peroxisomal biogeneses factor 7 (*PEX7*) genes with their SNPs showing strong association with different HbF levels [48]. It was shown that a short tandem repeat in the *MAP3K5* promoter, as well as intronic variations within both *MAP3K5* and *PDE7B* genes could be associated with lower HbF levels and thus, more severe β-thalassemia phenotype [49]. MAP3K5, encoded by *MAP3K5* gene, is a member of the MAPK family and, as such, a part of the MAPK pathway. This signaling cascade is one of the most important mechanisms for the cytoplasmic transduction of extracellular signals. As such, MAP3K5, has not been clearly associated with mechanisms governing erythropoiesis. On the other hand, *PDE7B* could be considered a strong modifier

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gene candidate given its high affinity and specificity for cAMP, which has an inhibitory effect on γ-globin expression and, thus, plays a role in fetal-to-adult globin gene switching [50].

3.2.2. BCL11A gene

BCL11A (B-cell CLL/lymphoma 11A) is transcriptional repressor expressed in most hematopoietic cells and critically important in the T and B cell development. BCL11A is a zinc-finger protein with usually a C2N2 zinc finger at the N-terminal, and six other Krüppel-like C2H2 zinc fingers near the C-terminal and it has at least 4 predicted isoforms (XS, S, L, XL) due to the alternative splicing. It is encoded by *BCL11A* gene, which spans over 102 kb on chromosome 2p16 [51]. Genome-wide association studies (GWAS) have demonstrated that a meaningful fraction of the variations in HbF levels is accounted for by variants within *BCL11A* gene [39]. More precisely, close to 15% of the phenotypic variation in the HbF levels could be explained by variations in intron 2 of the *BCL11A* gene, such as rs4671393 and rs11886868 [42, 43]. Further, it was shown that BCL11A (L and XL isoforms) acts as a potent silencer of γ-globin gene expression [52] by binding not to γ-globin gene promoter, but to LCR and γ^A-δ intragenic region with known role in repression of fetal globin genes [53]. Its role as a repressor, BCL11A most probably exerts trough association with various partners within erythroid multiprotein complexes, including the repressive nucleosome remodeling and deacetylase complex (NuRD), GATA1, the erythroid master regulator, and SOX6, a transcription factor previously shown to repress embryonic globin genes in mice [54]. Evidence that BCL11A acts as a γ-globin gene repressor also lays in a fact that *BCL11A* expression levels are much higher in adult compered to fetal developmental stage [54, 55]. As a direct repressor of fetal globin genes, BCL11A is the first genetically and biochemically validated regulator of the fetal to adult globin switch in humans [42] and, as such, represents potential target protein for HbF induction. However, since BCL11A acts as a transcriptional factor in non erythroid cells as well, recent studies have proposed GWAS-identified erythroid *BCL11A* enhancers as a particularly promising target for gene therapy in the β-thalassemia syndromes. This way, disruption of the *BCL11A* enhancers would only affect *BCL11A* expression in erythroid cells, while this gene expression would be intact in the non erythroid cells [56].

Genome-wide association studies indicate that variants in the *HBB*, *HSB1L- MYB* and *BCL11A* loci account for approximately 50% of the HbF level variation, suggesting that additional factors are involved [38, 39, 43, 57]. These factors may involve regulators of β-globin genes' expression (KLF1), proteins responsible for α-globin chain stability (AHSP) or factors involved in epigenetic regulation of fetal globin gene expression (FoP).

3.2.3. KLF1 gene

Erythroid Krüppel-like factor, (KLF1), previously known as EKLF, is one of the key erythroid-specific transcriptional factor that interacts with the CACCC box, important regulatory element of many erythroid genes, including adult β-globin gene and *BCL11A* [58, 59]. This interaction is carried out via three zinc finger domains, necessary for binding and activation of KLF1 target genes. KLF1 also contains proline-rich transactivation domain tough which this transcriptional factor preferentially activates the *HBB* gene at the expense of *HBG1*/*HBG2* gene

expression by interacting directly with regulatory elements in *HBB* promoter [60-62]. Proline-rich region contains functionally distinct activation (AA 20-124) and inhibitory domains (AA 195-291). The minimal activation domain (AA 20-124) can be further divided into a subregion (AA 20-60) that itself does not activate, but interacts trough intermolecular interactions with another cellular protein, providing optimal transactivation potential to its adjacent (AA 60-124) sequence. At the same time, the inhibitory domain (AA 195-291) operates intramolecularly, preventing efficient binding of the DNA-binding zinc finger region within the same molecule [63]. Mutations affecting any of these functions of the KLF1 transcriptional factor, could potentially, directly or indirectly, alter expression of β-globin genes.

The direct association of mutations in human *KLF1* with hemoglobin regulation was first described by the Borg *et al.* [59], who reported a single point mutation in *KLF1* gene (p.K288X) responsible for completely abolishing the DNA binding domain resulting in KLF1 haploinsufficiency. Results from this study showed that this reduced KLF1 activity results in decreased *BCL11A* expression and hence, diminished synthesis of BCL11, γ-globin gene repressor. This, in turn, leads to increased HbF levels, observed in the carriers of p.K288X mutation [59, 64]. Similarly, novel *KLF1* mutation (c.914-4_914-1 del CTAG), hypothesized to affect splicing, causes haploinsufficiency of *KLF1*, leading to reduced expression levels of KLF1 target genes [65]. Results form our study on the first *KLF1* promoter mutation also go in favor of a KLF1 being a potent regulator of HbF levels [66]. Namely, results form this study suggest that this KLF1:g.-148G>A mutation leads to reduced *KLF1* gene transcription, which could explain, at least in part, the observed HPFH phenotype, further underlining the significant role of KLF1 on human fetal globin genes switching [66, 67].

Number of mutations affecting *KLF1* gene, as well as our understanding of different phenotypes associated with these mutations, are very obscure [68]. However, studies on *KLF1* gene mutations, including the ones without an obvious phenotype, contribute to the better understanding of the human erythropoiesis in general [59, 69-71].

3.2.4. AHSP gene

The αHb-stabilizing protein (AHSP), also known as erythroid-associated factor (ERAF), is erythroid-specific protein with an important role in erythropoiesis. Namely, it is involved in folding of the α-globin chains for β-globin association, heme binding, transfer for β-globin association and stabilization of α-globin chains. AHSP specifically binds multiple forms of α-globin including the apo form (no heme present) and α-hemoglobin (αHb) (α-globin with heme). Its role as a specific molecular chaperone that binds α-globin chain of hemoglobin preventing its precipitation, imply that alterations in *AHSP* gene expression or protein function could influence β-thalassemia phenotypes [72-74]. While some studies reported that reduced expression of *AHSP* was associated with a more severe phenotype among individuals with identical β-thalassemia and α-globin [75, 76], others indicated that AHSP is not a disease modifier of β-thalassemia [77, 78]. Research on *AHSP* gene mutation showed that structural mutations are uncommon, and therefore, not likely to be major modifiers of β-thalassemia. However, it remains a possibility that rare *AHSP* null or missense mutations, genetic or epigenetic factors unlinked to the gene, could modulate coexisting β-thalassemia [73, 77].

3.2.5. FoP protein

Friend of Prmt1 (FoP) is a small arginine/glycine rich protein encoded by the *C1orf77* gene. This protein, through its association with protein arginine methyltransferase 1 (PRMT1), is involved in transcriptional regulation of globin genes via histone methylation [79]. Studies have shown that this protein is a critical modulator of HbF levels, since knockdown of this factor leads to elevated γ-globin gene expression. Although it is unclear how FoP regulates γ-globin gene expression, it is presumed that this induction of fetal globin genes occurs trough modulation of SOX6 which acts as BCL11A cofactor, and not trough BCL11A itself. These results identify FoP as a novel potential therapeutic target in β-thalassemia syndromes, as well as in other hemoglobin-related disorders [80].

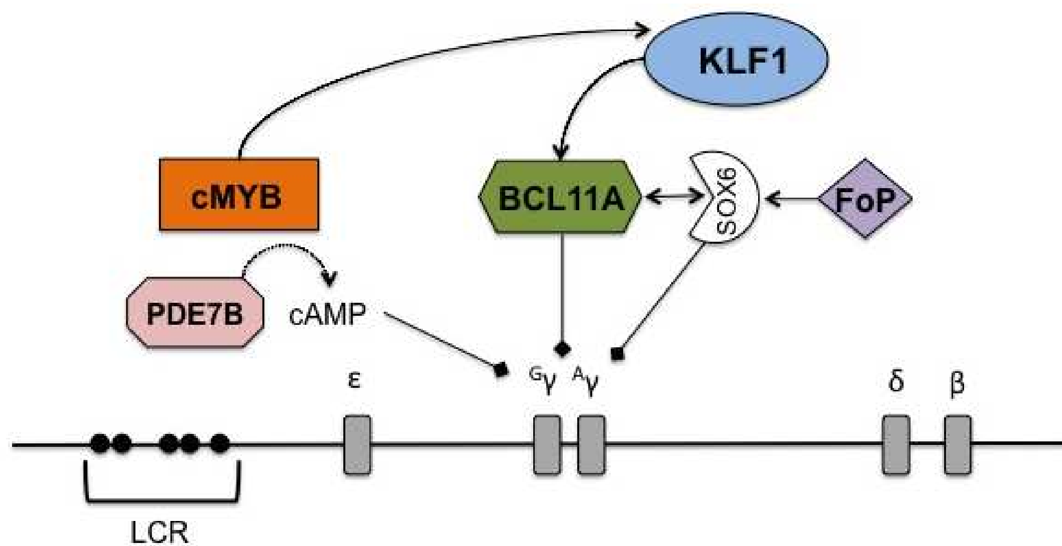


Figure 2. γ-globin gene regulators as potential targets for therapeutic induction of HbF. Human β-globin gene locus showing embryonic, fetal and adult globin genes, controlled by LCR. Transcription factors involved in regulation of γ-globin genes are shown. A line with an arrow at the end denotes positive regulation (activation). A line with a black box at the end denotes negative regulation (repression). Dashed line denotes ambiguous interaction. A line with arrows at two end points denotes mutual interaction. For details see the text.

4. Gene therapy

Despite immense achievement in the traditional care and treatment of β-thalassemia, including transfusion and drug therapy, until recently, a definitive cure for these disorders could only be achieved by bone marrow transplantation (BMT) from related or unrelated donors. However, BMT is available for only a small fraction of β-thalassemia patients and is characterized by relatively high mortality and morbidity, especially in the case of unrelated donors [27]. As an answer to these limitations of BMT and the need for more permanent solution, arose transfer of a therapeutic gene using autologous hematopoietic stem cells (HSC) as potential definitive cure for β-thalassemia syndromes.

The goal of this gene therapy is substitution of a defective or missing protein by introducing an intact copy of the faulty gene in question or by introduction of a gene which modifies the effect defected gene has on a cell such as β-thalassemia gene modifiers do.

For the successful gene therapy for β-hemoglobinopathies, several requirements need to be met. Those include high-efficiency gene transfer and high HSC engraftment; consistent levels of β-globin gene expression, independent of the site of integration; high expression levels of β-globin or γ-globin genes; regulated expression in the erythroid lineage; safe expression with little or no risk of insercional mutagenesis/oncogenesis [81].

Hematopoietic stem cells, as the targets for gene transfer, are harvested from patient bone marrow or peripheral blood following cytokine mobilization, purified by immunoselection, transduced and returned to the patient. Various types of vectors have been considered for gene transfer, including viral and non viral vectors [82].

4.1. Non viral vectors

Indication that gene therapy, as a therapy of the future for thalassemia syndromes, could be possible, came from the study on suppressor tRNA [83]. In this study a human tRNALys gene was converted to an amber suppressor by site-specific mutagenesis of the anticodon. As a result, a tRNA that suppressed the UAG amber nonsense mutation in β^0-thalassemia mRNA was produced giving rise to functional β-globin polypeptide. Although promising, the use of such genes in gene therapy would be limited to β^0-thalassemias due to nonsense mutations. Also, amber suppressor tRNA could suppress termination of the proteins with UAG as their normal termination codon making these genes not an ideal candidate for use in gene therapy.

As a potential approach to gene therapy for hemoglobin disorders, the catalytic properties of ribozymes to alter the defective mRNA produced by the mutated β-globin gene was also explored [84]. Namely, *trans*-acting group 1 ribozyme was created in a such a way that it was able to convert mutated β-globin transcript into RNA encoding γ-globin. This way, not only would the mutated gene be removed but it would be replaced with gene producing fetal globin chain. Although these results were very promising, several problems emerged, one of which is long-term effect of the therapy since it is directed at transcription product and not at the mutated gene itself [85].

Contrary to these non viral vectors, viral vectors showed higher gene transfer efficiency, since they do not require harsh physical means, such as electroporation, to enter the cell [86].

4.2. Viral vectors

The first viral vectors used to transfer the human β-globin gene in the mouse HSCs were oncoretroviruses or gamma-retroviruses (γRV), which efficiently transfer therapeutic gene into HSCs without transferring any viral genes. Gamma-retroviruses belong to the family of Retroviridae, which, among others, also include lentivirus (LV) and foamy virus (FV), both used in clinical trials or preclinical testing. These viral vectors are constructed in such a way that the genetic elements needed for pathogenicity and replication are removed and are replaced with the cellular transgene of interest [86, 87].

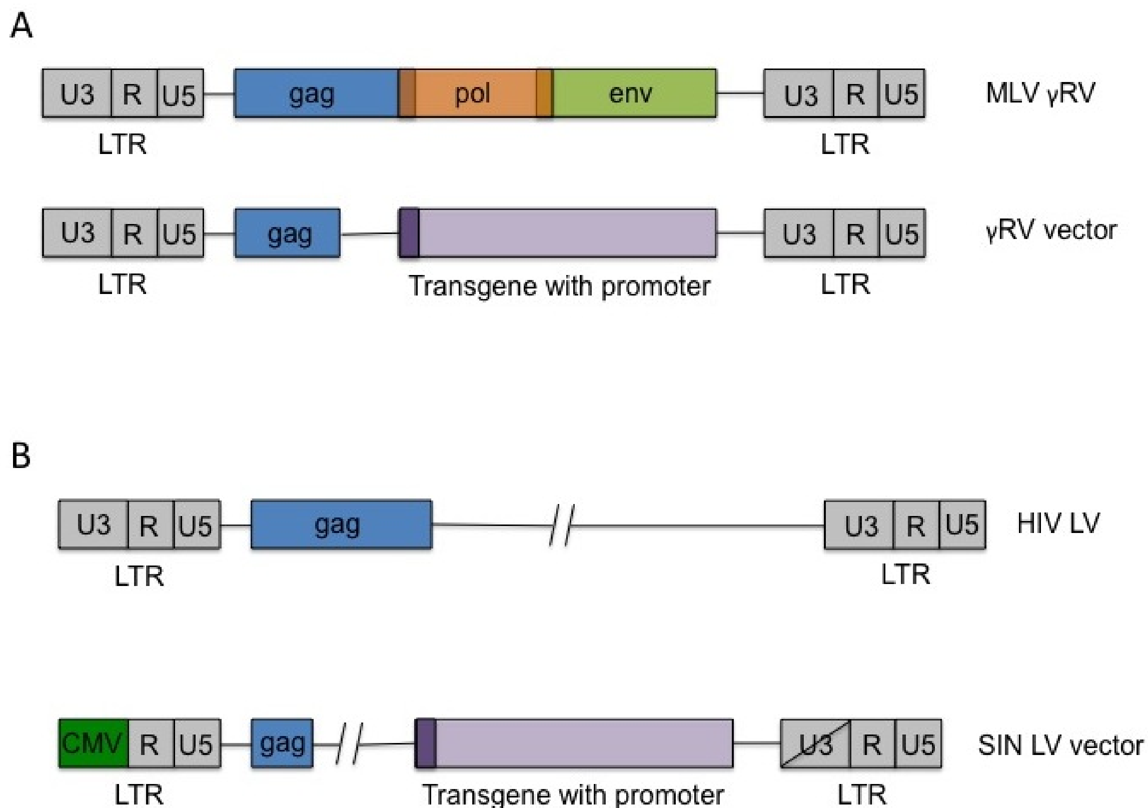
4.2.1. Retroviral vectors

The γRV vectors contain intact viral long terminal repeats (LTRs) which enclose U3, R and U5 regions. The U3 region has strong promoter and enhancer activity and is usually used by the virus to reverse transcribe and incorporate the genetic material into the host genome (Figure 3.) [86]. Early attempts to transfer human β-globin gene in the mouse HSCs using these vectors, resulted in tissue-specific, but low and variable human β-globin expression in bone marrow chimeras, usually varying between 0% and 2% of endogenous mouse β-globin mRNA levels [88]. In order to increase expression levels of transferred β-globin genes, efforts were made to include LCR elements of the β-globin gene locus into these γRV vectors. Incorporation of the LCR's DNase I hypersensitivity sites, HS2, HS3 and HS4, significantly increased expression levels in the murine erythroleukemia (MEL) cells, but failed to abolish positional variability expression and resulted in vectors with low titers [81, 87]. Gamma-retroviral vectors were also limited by their size, need for cell division before integration, as well as their stable transmission since they were so unstable that the transduced globin gene was very rarely integrated intact into the genome, thus losing any therapeutic efficacy [27].

4.2.2. Lentiviral vectors

Bioengineering of HIV-1 devoid of any pathogenic elements resulted in the development of lentiviral (LV) vectors as suitable vectors for high-efficiency gene transfer. In order to improve its safety, self-inactivating (SIN) LV vector was constructed by deleting the viral promoter/enhancer in the U3 region of the 3′ LTR, without significant loss in titers or infectivity. During reverse transcription of the viral RNA, this deletion that gets copied to the 5′ LTR, minimizing transactivation of neighboring cellular promoters, thus improving the safety of the vector itself [89]. Instead of U3 region in the 5′ LTR, SIN LV vector contains a cytomegalovirus (CMV) promoter which is only used in packaging the vector and is not transmitted to the host cell (Figure 3.). This new generation of LV vectors display variety of advantages compared to γRV vectors including the ability to infect quiescent, nondividing long-term HSC (HSC capable of self renewal). Unlike γ RV vectors, SIN LV can stably carry larger and more complex transgene cassettes containing introns and regulatory elements, necessary for high globin gene expression. LV vectors usually insert these cassettes within gene introns, avoiding promoters and 5′ regulator regions which RV vectors have high affinity for [86, 90].

With the discovery of LV vectors as a potent transporter of gene of interest, research turned to the globin cassette itself. The first study to demonstrate stable transmission and high-level β-globin gene expression in a mouse model of β-thalassemia intermedia, tested two types of LV vectors. Larger, TNS9 vector, contained large LCR fragments encompassing HS2, HS3 and HS4 and was approximately 3.2 kb in size, while smaller, RNS1 vector carried only a minimal core LCR elements. It was shown that cells transduced with TNS9 vector sustained higher human β-globin transcript levels. Also, this vector achieved significant improvement in the hematocrits, red blood cell and reticulocyte count, as well as hemoglobin levels in β-thalassemia mice [91]. Today, vectors' globin cassette usually contains β-globin gene with deleted destabilizing RsaI fragment located within intron 2 and several sequences surrounding HS of the LCR. However, the number and the length of the HS sites vary in all globin cassettes, since



For details see the text.

Figure 3. A. Genome organization of MLV γRV and γRV vector; B. Genome organization of HIV LV and SIN LV vector.

it's proven very difficult to define really important regulatory sequence within each HS, while omitting potentially destabilizing elements. Also, some of the vectors in use are flanked by insulators, genomic element that can shelter genes from their surrounding chromosomal environment, resulting in position-independent expression [27].

5. Gene therapy ofβ -thalassemia: Success or fail?

The first successful human gene therapy for β-thalassemia was achieved in 2007., when HbE/β^0-thalassemia major patient was transduced with vector containing antisickling β-globin (βA(T87Q)), a 260 bp long globin promoter, HS2, HS3, HS4 and two copies of insulator flanking the globin cassette. Although the patient became totally transfusion independent, it was discovered a dominant cell clone with integration site into the *HMGA2* gene, a potential oncogene. While it was shown that overexpression of *HMGA2* is mainly associated with benign tumors, this observation points out some of the limitation of lentiviral vector gene transfer, which include the need for improved efficiency of gene delivery and insertion of the gene into non-oncogenic sites [92].

http://dx.doi.org/10.5772/61023

As the secondary modifier genes begin to take a center stage in the fight against β-thalassemia syndromes, researchers turned to these genes as potential target genes in gene therapy for β-thalassemia. Namely, transduction of CD34 cells with lentiviral vector carrying a short hairpin RNA (shRNA) targeting the γ-globin gene repressor BCL11A, led to significant increase of HbF in differentiated erythroblasts, around 10% in cells derived from normal donors and from 33% to 45% in β-thalassemic cells [93].

In the past few years, induced pluripotent stem cells (iPSCs) emerged as an interesting candidate for gene transfer. iPSCs are generated from mature somatic cells derived from skin fibroblasts, amniotic fluid or chorionic villus of β-thalassemia patients, by transduction with number of specific transcriptional factors. More specifically, it was shown that fibroblasts derived from tail-tip of a mouse, homozygote for human sickle cell allele (β^S/β^S), and infected with retroviruses encoding for Oct4, Sox2, and Klf4 factors, as well as with a lentivirus encoding a 2-lox c-Myc cDNA, were transformed into iPSC. These iPS cells, after being infected with an adenovirus encoding Cre-recombinase to delete the lentivirus-transduced c-Myc copies, in order to reduce the potential risk of tumor formation due to c-Myc transgene expression, stained positive for pluripotency markers. They also had a normal karyotype, and generated teratomas and chimeras. iPSC obtained in such a way were later successfully used in specific correction of the sickle cell allele, by being electroporated with a construct containing the human wild type β-globin gene. [94].

iPSCs are easily obtainable and represent an endless source of stem cells for gene manipulation and correction strategies. Therefore, the major advantage of these cells, when it comes to gene therapy, is the possibility to screen and choose the ideal clone with safe integration and high transgene expression profile. Unfortunately, several obstacles stand in the way of iPSCs being successfully used in gene therapy, one of which is elimination of the transcriptional factors used for induction, once they are no longer needed. More importantly, it is necessary to establish the correct re-programming so that the iPSCs do not develop tumors [27, 86, 87].

6. Conclusion

The β-thalassemias are the best understood disorders at the molecular level. Accordingly, therapy protocols based on molecular basis of β-thalassemia have been designed as an example of novel approaches in disease treatment. However, there is no well established gene therapy protocol for β-thalassemia to date. Why is so difficult to design an appropriate “therapeutic gene”, even for this monogenic disorder, and to deliver it to hematopoietic stem cells to achieve therapeutic effect in β-thalassemia patients? Despite extensive research, modern science does not understand a complex gene expression regulation of globin genes yet. First of all, there is very specific regulation of expression of globin genes during ontogenesis. Also, tissue (erythroid)-specific regulation is present. Moreover, globin genes are regulated in a cell-specific manner since they are expressed only in the certain stages during differentiation of erythroid cell lineage. Finally, there is coordination between expression of α- and β-globin genes.

Besides all that, our knowledge is accumulating, and there is no doubt that gene manipulation will begin to cure in near future. Certainly, only somatic gene therapy is considered, since germ-line gene therapy raises many unique ethical concerns. Hopefully, thanks to gene therapy, a large number of people suffering from β-thalassemia will have a long and better life, despite the predispositions. That way, an old proverb will finally become true: "Fato prudentia maior est" (Wisdom is stronger than destiny).

Acknowledgements

This work was supported by Ministry of Education, Science and Technological Development, Republic of Serbia (Grant No. III41004).

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