Chapter 7

Modifiers of γ-Globin Gene Expression and Treatment of β-Thalassemia

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Abstract

Beta thalassemia (β-thalassemia) is an autosomal recessive genetic disease with many genes involved. It is a heterogeneous disorder caused by variations in the inactivation mechanism of the Beta-globin (β-globin) genes. Despite seemingly similar genotypes, the patients with Beta-thalassemia have a remarkable variability in anaemia, growth development, and hepatospleenomegaly and transfusion requirements. The genetic factors may differ in each race or ethnic group for therapy and prevention. Despite remarkable successes in the treatment of Beta-thalassemia in the past decades, it is still the leading cause of death and premature disability in developed and developing countries. Possible factors that influence the severity of anaemia in thalassemia may be inherited or non-inherited. The inherited factors include the type of β-thalassemia, coinheritance of alpha thalassemia (α-thalassemia) and factors that stimulate fetal hemoglobin (HbF) production. In this chapter, respective contributions of known modifiers and also the pharmaceutical agents currently in use and under clinical trials for regulating the globin gene expression will be discussed.

Keywords: Hemoglobinopathies, gamma globin, fetal hemoglobin, modifier genes, drug discovery

1. Introduction

1.1. General introduction

The thalassemias are the commonest monogenic disorders in the world, and globally it is estimated that there are 270 million carriers, of which 80 million are carriers of β-thalassemia. β-thalassemia is widespread in the Mediterranean, Southeast Asian, African, and Middle East populations. The mean prevalence of this disease in India is 3.3%. It has become much more common recently in northern and central Europe, including Germany, due to immigration [1–

3]. The thalassemias refer to a diverse group of hemoglobin disorders characterized by a reduced synthesis of one or more of the globin chains (α, β, γ, δβ, γδβ, δ, and εγδβ). β-thalassemia occurs when there is a deficiency of β-globin, which is typically caused by a direct down-regulation in the synthesis of structurally normal b chains. However, a thalassemia phenotype can also arise from structural b chain variants if they are synthesized at a reduced rate. The most severe form of thalassemias is characterized by the complete absence of HbA ($\alpha_2\beta_2$) which results from the inheritance of two homozygous β-thalassemia alleles. This normally presents as a life-threatening anemia requiring blood transfusions from infancy. Inheritance of single β-thalassemia alleles is presented by a clinically asymptomatic condition, but may show a mild anemia [4].

More than 200 mutations of the β-globin gene which can lead to β-thalassemia major have been characterized worldwide [5–8]. Deletion mutations are rare; the majority of mutations are point mutations in important functional regions of the β-globin gene [9]. Splice mutations and those that occur in the promoter region tend to cause a reduction, rather than a complete absence of β-globin chains, and thus result in milder disease. Nonsense mutations and frameshift mutations tend not to produce any β-globin chains leading to severe disease (loss of function). The severity of anemia caused by β-thalassemia depends on which mutations are present and on whether they decrease beta-globin production (called beta$^+$ thalassemia) or there is complete elimination (called beta^0 thalassemia). A subset of common mutations is present in each ethnic group in which the disease is prevalent.

2. Clinical heterogenity in β-thalassemia

Clinical manifestations of β-thalassemia are extremely variable, ranging from nearly asymptomatic to severe and transfusion-dependant [10]. However, the marked variability of the clinical manifestations is not well-understood till date. The clinical picture in some patients may be a devastating course of acute and chronic events, resulting in severe organ damage. In other patients, the disease may be present with relatively mild clinical phenotype and minimal morbidity [11].

3. Factors influencing clinical severity

Various factors have been found to modulate the beta-thalassemia phenotype. These include the presence of alpha-thalassemia, elevated HbF levels, haplotypes that are linked to the beta-globin gene and chromosomal sites different from chromosome 11 [12]. Co-inheritance of a single alpha-globin (α-globin) gene deletion with β-thalassemia may have a milder phenotype, whereas deletion of both α-globin genes is typically associated with thalassemia intermedia [13]. Fetal hemoglobin is one of the most common and major modifiers of the disease severity in individuals with β-thalassemia. Because the severity of homozygous β-thalassemia is directly related to the imbalance between α- and β-globin chains, even the low levels of γ-

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globin in F cells reduce the relative excess of α-globin and provide a selective survival of cells making HbF at the time of ineffective erythropoiesis, in the most severe forms of β-thalassemia. Any factor that can reduce the degree of imbalance (by reducing α or increasing β and/or γ globin chains) may ameliorate the clinical phenotype [14]. Therefore, this selective survival might account for elevated levels of HbF in homozygous β-thalassemia. The presence of genetic variants that sustained fetal hemoglobin production have a strong impact on ameliorating the clinical phenotype.

4. Elevated levels of fetal hemoglobin (HbF)

HbF ($\alpha_2\gamma_2$) accounts for up to 90% of the total hemoglobin at birth. Its synthesis starts to decline during the third trimester, and over the first year of life, it is gradually replaced by adult Hb ($\alpha_2\beta_2$). An elevated level of HbF in an adult may result from a genetic disorder of hemoglobin production or from various acquired hematological conditions (such as placental insufficiency) in newborn infants of diabetic mothers, such as severe anemia, bronchopulmonary dysplasia (BPD), and congenital cyanotic heart (CCHD) [15–18]. Erythropoietic stress and hypoxemia has also been shown to increase the production of HbF [19–21]. Additional γ-globin chains bind with the excess of α-chains and decrease the imbalance between α- and β-globin chains caused because of absence of beta-globin chains, decreasing the deleterious effect of intracellular precipitation of unbound β-chains [22–23]. This reduces ineffective erythropoiesis and some functional HbF is produced in the red cell precursors which help red blood cells survive longer duration in the circulation. Induction of fetal haemoglobin expression in erythroid cells is an important therapeutic approach in patients with haemoglobin disease [24-25].

5. Genetic modifiers of γ-globin gene expression

To understand the clinical or molecular relationships, it is important to remember that the main pathophysiological determinant of the severity of beta-thalassemia is more than that of alpha/non-alpha-globin chain imbalance. Therefore, any factor capable of reducing the alpha/non-alpha-globin chain imbalance may have an ameliorating effect on the clinical picture [7]. Certain mutations within the β-globin promoter region are associated with increased γ-chain expression from the same chromosome [7]. These are known as primary modifiers. Recently, genetic variants that modulate HbF levels, but fall outside of the Hb genes (Secondary modifiers) have also been identified [26]. These modifiers, known as secondary modifiers, primarily act directly to alter the known pathophysiology of the disease.

5.1. Primary modifiers

Primary modifiers have been identified as different mutations within the β-globin gene resulting in varying severity. Severity of β-thalassemia ranges from the mutations (β0) causing complete absence of β-globin production to those in which β-globin is produced but to a small extent (β+). Inheritance of two β+ genes such as -28 ATA box (A → G) codon 19 (A → G); -90

C → T, -88 C → A; -88 C → T, -87 C → A; -87 C → G, -87 C → T; -86 C → G, 31 A → G results in a milder disease [27].

Some β-thalassemia mutations are completely silent and have been shown to cause a milder form of the disease. The most common is the -101 (C → T) mutation which interacts with different severe β-thalassemia alleles to produce a mild form of the disease [28]. Co-inheritance of severe β-thalassemia allele (β^0) with hemoglobin E (HbE) allele also results in variability of the phenotype. Alternative to the splicing of HbE globin pre-mRNA, the amount of mRNA spliced may also play a role in the phenotypic variability.

5.2. Secondary modifiers

Secondary genetic modifiers affect the clinical severity of the disease by reducing the globin chain imbalance, therefore resulting in a milder form of the disease phenotype [29]. Genome-wide studies (GWAS) have shown the association of genetic polymorphisms in three major loci Xmn1-HBG2, HBS1L-MYB intergenic region (HMIP) on chromosome 6q23, and BCL11A on chromosome 2p16, accountable for a relatively large proportion of the phenotypic variation in HbF levels. The gene encoding for zinc fingers and homeobox 2 (ZHX2) transcription factor located on 8q24 has emerged as a potential candidate gene for γ-globin regulation.

- Xmn-1 (Xanthomonas maniholis-1) Restriction Site

The presence of one polymorphism Xmn-1 restriction site at -158 position of the Gγ gene has been co-related with the increased production of HbF in adults under haematopoietic stress. A specific (C → T) mutation at the -158 position in the promoter region of the Gγ gene creates a restriction site for the Xmn-1 endonuclease. Higher expression of the fetal hemoglobin (HbF) in adulthood has been indicated to ameliorate the morbidity and mortality in sickle cell disease (SCD). Nemati et al. have studied the frequency of Xmn1 polymorphic sites in β-thalassemia major patients from Western Iran. The study revealed that the presence of this polymorphic site caused a positive influence on HbF production and the G-γ percent, which could improve the clinical symptoms of β-thalassemia patients [30]. In another study carried out by Pandey et al., the phenotypes of Indian sickle cell patients were found to be greatly influenced by Xmn1polymorphism [31].

In a recent study, we have evaluated the association of Xmn1 polymorphism with mild, moderate, and severe groups of β-thalassemia as well as sickle cell anemia (SCA). A significant association of TT genotype and T allele was observed with milder disease phenotype. In addition, we also evaluated the association of Xmn1 polymorphism with HbF levels to estimate whether this variant modifies the phenotype of homozygous β-thalassemia as well as SCA by modulating the HbF levels. A significant difference in the high and low percentage of HbF in CC, CT, and TT bearing individuals ($P<0.01$) were observed. This study confirms that increased γG-globin expression, associated with the Xmn1 polymorphism ameliorates the clinical severity in β-thalassemia as well as SCA in the study population [32].

- Zinc-fingers and homeoboxes 2 (ZHX2) gene

The gene coding for ZHX2 or KIAA0854 in human is located on chromosome 8q24.13 in the position 123863082-124055936. Four exons code for 837 amino acid protein of approximately

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92 kDa. The ZHX2 mRNA is expressed among various tissues. ZHX2 is a transcriptional repressor [33] and has been identified as a factor which is involved in postnatal repression of fetal expressing genes [34]. Therefore, ZHX2 is a novel candidate gene for globin regulation in erythroid cells [35]. In 2005, Perencheri et al. indicated that overexpression of a ZHX2 transgene restores H19 repression on a BALB/cj background, confirming that this gene is responsible for hereditary persistence of the α-fetoprotein (Afp) and H19 which are transcribed at high levels in the mammalian fetal liver but are rapidly repressed postnatally [36]. Down-regulation of ZHX2 was recently demonstrated in two HPFH-2 subjects by real-time PCR [35].

ZHX2 gene coincides on the quantitative trait loci (QTL) on chromosome 8q that has been reported to influence the absolute fetalhemoglobin levels [37]. Taken together, ZHX2 is a good candidate gene for regulating γ-globin gene expression. Aussara Panya et al. demonstrated the effect of ZHX2 overexpression and the down-regulation of γ-globin expression in K562 cells [38]. Further, their study supported that ZHX2 is involved in the repression of the γ-globin gene, but also provides evidence that ZHX2 has a direct effect on the γ-globin expression level and might participate in the globin gene regulation [38]. In another study, de Andrade et al. reported the marked down-regulation of ZHX2 in CD34+ cells cultured in a high fetalhemoglobin-enriched condition [39].

- B-cell lymphoma/leukemia 11A (BCL11A) Gene

The BCL11A gene is essential for normal lymphoid development [40] and has been associated with hematologic malignancies [41]. The BCL11A gene was initially identified from aberrant chromosomal translocations involving the immunoglobulin-heavy chain locus detected in B-cell non-Hodgkin lymphomas [42]. It encodes a kruppel-link zinc finger protein containing 3 C_2H_2 zinc finger motifs, a proline-rich region, and an acidic domain. BCL11A gene located on chromosome 2p16.1 is expressed predominantly in the brain, spleen, and testis.

A study has demonstrated that an intronic SNP in BCL11A, rs11886868 strongly co-relates with HbF levels in a large cohort of sickle cell patients [43], this indicates that BCL11A variants, by modulating HbF levels, act as an important ameliorating factor of the SCA and it is likely they could help ameliorate other hemoglobin disorders. Genotyping of two other SNPs rs4671393 and rs7557939 in the Brazil Sickle cell disease cohorts have shown that these SNPs were more strongly associated with HbF level variation than rs11886868 [44].

In a recent study, we evaluated the association of rs11886868 of the BCL11A gene with mild, moderate, and severe groups of β-thalassemia and SCA. A significant association of CC genotype and C allele was observed with milder disease phenotype in β-thalassemia as well as SCA [45].

- Friend of Protein (FOP)

Friend of protein arginine methyltransferase I (FOP) has been reported to be one of the targets of protein arginine methyltransferase 1 (PRMT1), which stably interacts with chromatin [46]. Initially this protein was studied for its role in estrogen-dependent gene activation and in hemoglobin switching [46]. Studies have shown that knockdown of FOP in murine fetal liver cells transgenic for the β-globin locus significantly increased expression of both the mouse

embryonic and human adult peripheral blood erythroid progenitors resulting in up-regulation of γ-globe and increased production of HbF [46]. As a result of this, HbF levels were reported to increase from background levels of 6% to 7% to an average of approximately 27%. It was observed that BCL11A levels were not altered interestingly, but levels of Sex determining region Y-box 6 (SOX6) protein appeared to be reduced. Therefore, there is a possibility that FOP depletion increases globin expression via reduction of SOX6.

- Sex determining region X-box 6 (SOX6 -SRY)

SOX6 plays an important role in the silencing of the embryonic globin genes. This gene encodes a member of the D subfamily of sex determining region y-related transcription factors. It is characterized by a conserved DNA-binding domain termed the high mobility group box and has the ability to bind the minor groove of DNA. The encoded protein is a transcriptional activator which is required for normal development of the central nervous system, chondrogenesis, and maintenance of cardiac and skeletal muscle cells. This protein interacts with other family members to activate gene expression. Alternative splicing leads to multiple transcript variants. First of all, SOX6 was observed to play a role in the silencing of the mouse embryonic globin genes. It binds to the murine γ-globin promoter as observed by Chromatin immune-precipitation [47]. It was also found that SOX6 interacts with BCL11A, although SOX6 strongly binds the globin gene promoters suggesting that the SOX6-BCL11A protein-protein interaction may involve chromatin looping [48]. SOX6 binding to the γ-globin promoters requires BCL11A. Studies have shown that the knockdown of SOX6 in cultured, primary human erythroblasts led to a small up-regulation of HbF but when combined with BCL11A knock-down, further enhanced HbF production [48].

- KLF1

KLF1, known as Erythroid Kruppel-like factor, is a zinc finger protein which binds the CACCC of the adult globin gene in mice as well as humans [49–50]. This protein is important for its expression responsible for ablation in mice causing embryonic lethality due to severe anemia [51–52]. Studies have shown that both endogenous mouse embryonic and human fetal globin gene expression are not properly down-regulated in the *Eklf*$^{-/-}$ mice. The haemoglobin switching role of this protein was identified in further studies [53–54]. A GWAS scan followed by linkage analysis of a family including 27 members showed that 10 out of 27 members had Hereditary persistence of fetalhemoglobin phenotype. In this study, a nonsense mutation in KLF1 gene was identified, which ablated the DNA binding domain, resulting in haploinsufficiency in the heterozygous state [53]. The HbF levels were found to vary among individual family members, indicating the influence of other genetic and environmental factors. In addition to the binding globin gene promoter, normal KLF1 was reported to show strong binding to the BCL11A promoter, thereby activating its expression in cultured, primary adult human erythroblasts. KLF1 has been found to mediate its switching effects through a dual mechanism, acting both on the globin and BCL11A promoters. Zhou et al. also found that reduced, but not absent, KLF1 levels in genetically altered mice resulted in markedly decreased levels of BCL11A mRNA and protein [54]. The researchers found that there was a strong binding of KLF1 to the Bcl11A promoter. Further, mouse embryonic and human fetal globin

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transgene expressions were found to be highly increased in the setting of reduced KLF1. Similar to this, it was found that when KLF1 was knocked down in cultured adult human erythroblasts, γ-globin gene expression was up-regulated [54]. Therefore, both compelling evidence from genetic and functional data suggests that KLF1 has a significant role in the fetal to adult switch. However, a 2- to 3-fold elevated expression in adult versus fetal cells, which results in KLF1's preferential association with the β-promoter in the former and the γ-promoters in the latter, is still not clear.

- HBSL1-MYB DNA region

In a GWAS, designed to identify polymorphisms associated with the variability of HbF expression in nonanemic humans, variants in the intergenic region between the HBSL1 and MYB genes were identified [55]. Data suggests that the intergenic area is most highly associated with HbF expression and consists of properties of a regulatory element [56]. The linkage to an equilibrium block, which is most closely associated with HbF levels, is between the HBS1L and MYB genes, which are present on opposite DNA strands. Three hypersensitive sites have been found within that region. These sites have been recognized as the characteristic marks of active chromatin, including histone acetylation and RNA polymerase II binding in erythroid, but not in nonerythroid cells [56]. HBS1L is supposed to be a housekeeping gene because it is ubiquitously expressed. Whereas MYB has more of a restrictive pattern of expression and is crucial for erythroid development.

Studies have compared the expression profiles of 5 genes within this region, including MYB and HBS1L, in the erythroid cultures of 12 persons with elevated and 14 persons with normal HbF levels [57]. In this study, both MYB and HBS1L were down-regulated in persons with elevated HbF levels, whereas the other 3 genes did not change in their level of expression. Overexpression of MYB inhibited-globin gene expression in human erythroleukemia cells, whereas overexpression of HBS1L was reported with no effect. The researchers speculated that low levels of MYB result in fewer cell-cycle events early in erythropoiesis and that early maturation of erythroblasts yields red cells containing higher levels of HbF [57]. Overall, MYB has a critical role in erythropoiesis, and studies suggest that it acts, in part, by transactivation of KLF1 and LMO2 expression. 84 studies involving genotyping and resequencing have found that rare missense mutations might provide further evidence for the involvement of MYB in modulating HbF levels [50].

- TR2/TR4 DR erythroid-definitive complex

A repressor of the human embryonic ε-globin gene, the direct repeat (DR) erythroid-definitive complex consists of a heterodimer of 2 orphan nuclear receptors, TR2 and TR4 [58–59]. In vitro studies have found that this dimer, along with the nuclear factor COUP TFII binds DRs in the ε-globin gene promoter. Later, it binds to a similar repeat with the γ-globin gene promoters [60]. Endogenous murine embryonic to adult globin gene and fetal to adult human γ-globin transgene switching is delayed in mice which are null for TR2/TR4. In contrast to this, enforced expression of a dominant negative TR4 results in both loss of endogenous embryonic gene expression and human γ-globin transgene silencing. A mutation at -117 of the γ-globin promoter was indicated to be associated with HPFH, thereby affecting a DR element and

influencing factor binding, adding to the evidence that this heterodimer plays a role in stage-selective repression of the human γ-globin gene [61–62].

- COUP-TFII

COUP-TFII was known as one of the 2 factors required for transcription of the chicken ovalbumin gene [63]. COUP-TFII was later found to bind to the γ-globin promoter DR in vitro as well [64]. Mutation of DR elements resulted in derepression of γ-globin transgene expression in adult mice, indicating that DR sites are involved in fetal globin gene silencing. An in vitro model of primary human erythroblast development was used to show that the cytokine stem cell factor, through activation of the Erk1/2 and/or p38 mitogen activated protein kinase pathways, suppresses COUP-TFII expression at the mRNA and protein level, resulting in a large reduction in its binding to the γ-globin promoter.

- NF-E4

NF-E4 was identified as a component of the "stage selector protein," which binds to the stage selector element in the proximal-globin promoter in human K562 erythroleukemia cells; NF-E4, in conjunction with the ubiquitous transcription factor CP2, facilitates transcription of the γ-globin gene [65–66]. The stage selector element is conserved in species having γ-globin genes and is not present in species lacking HbF. The p22 NF-E4 acts as an "activator" of γ-globin transcription via interacting with CP2 to recruit p45 NF-E2 and subsequently RNA polymerase II to the promoter. Further evidence supporting a role for NFE4 in HbF production is that, an HPFH mutation at -202 creates a new binding site for the NF-E4-CP2 complex [67]. This alternate form, p14 NF-E4, apparently generated by translational initiation from an internal methionine, has been suggested to play a role in γ-globin gene down-regulation or silencing. The smaller isoform is abundant in adult erythroid cells, and although it did not bind the-globin promoter, it appears to interact with CP2 and might function to sequester NF-E2, which prevents complexing of activating factors at the γ-globin promoter. Enforced expression of p22 NF-E4 in transgenic mice harboring the human β-globin locus delayed the switch, without elevating the expression of HbF in the adult [67].

- GATA-1

GATA-1 is a member of zinc finger transcription factor and plays a seminal role in the development and differentiation of many cell types, including mega karyocytes, erythrocytes, eosinophils, and mast cells [68–69]. β-globin locus transcriptional activation can be resolved into discrete molecular steps involving the formation of distinct GATA-1 cofactor assemblies at the promoters and the Locus control region (LCR) [70]. Data suggest that GATA-1 can act both as a repressor and activator of gene transcription. Interaction between GATA-1 and the YY1 protein is involved in the silencing of the ε-globin gene in primate and non-primate species [71]. Moreover, binding of a GATA-1–FOG-1–NuRD complex was indicated to silence hematopoietic genes in erythroid cells [69, 72].

GATA-1 has multiple partners of proteins, including Stat3, Stat5, FOG-1, TAL-1, and Gfi-1b among others [73–75]. GATA-1 was firstly identified as a transactivator [73], however, its ability to mediate gene repression can be achieved through different DNA binding sequences,

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e.g. palindromic GATA-1 motifs and double GATA sites [76–77]. GATA-1 repressor activity has been implicated in naturally occurring mutations in the γ-globin genes which are associated with the persistent HbF synthesis after birth. These include point mutations at base -175 (T → C) and -173 (T → C) of the γ-globin promoter, which reduces GATA-1 binding and produces elevated HbF levels in humans and transgenic mice respectively [78]. Likewise, the -567 and -566 GATA-1 sites in the Gγ- and Aγ-globin promoter, respectively, have been found to be associated with gene silencing [79–80]. Amrolia et al. defined a repressor element in the γ-globin 5′-UTR, which was bound by a complex of two proteins, identified as GATA-1 and a ubiquitous negative regulator [81]. In a study, it was suggested that Stat3 binds the γ-globin 5′UTR to silence γ-globin transcription while GATA-1 binds the same region to enhance γ-promoter activity. Collectively, these studies suggest a model whereby GATA-1 acts as either a direct repressor or interacts with factors that bind nearby canonical sites to silence gene expression as proposed by Amrolia and co-workers [81].

In support for data provided by Harju-baker et al., a T3G mutation in the 567 GATA site of the gamma-globin gene in an Iranian-American family was reported to be associated with an HPFH phenotype by David Chui [80].

5.3. Induction of γ-globin gene expression

High HbF levels modulate the phenotype of the disease and ameloriate the severity of β-thalassemia and related complications led to an idea that reactivation of γ-globin gene will be a promising approach for the treatment of the disease [82–83]. Treatment strategies can be based on the following factors: an understanding pathophysiology of the disease; perspective of HbF to alter its manifestation; and that the developmental changes in γ-globin gene can be reversed by controling cellular and molecular regulatory mechanism [21]. Different classes of HbF inducers for clinical use have been reported.

Studies have shown that there are a number of inducers of the gamma-globin gene, like epigenetic modifiers of HbF and HbF inducers from the natural world.

- Epigenetic modifiers

If the molecular events occurring during haemoglobin switching are better understood, HbF expression could be more fully reactivated in adult cells and might lead to a cure for β-thalassemia.

The expression of human β-like globin genes are tissue- and developmental stage-specific in vivo. The mechanisms involved in the regulation of globin gene expression have been the subject of consideration for many years. Studies have led to the identification of cis- and trans-acting factors that regulate the expression of the β-globin gene cluster. Such studies also led to the recognition of the important role of epigenetic modifiers of globin gene expression. DNA methylation and histone acetylation are two of the most important epigenetic modifications that are involved in the regulation of most of the eukaryotic genes, including the genes of the globin family. Thus, targeting epigenetic silencing of the fetal globin genes can be a novel therapeutic approach for patients with β-thalassemia.

Epigenetic modifiers of HbF can be grouped in several classes, with different mechanisms of action. Different epigenetic modifiers of γ-globin gene are namely: erythropoietin, short chain fatty acids and cytotoxic agents, azacytidine, and hydroxycarbamide [84] (Table 1).

Many inducers inhibit histone deacethylase (HDAC) activity [85]. While most of the inducers exhibit inhibitory effects on cell growth, very few of them trigger increased γ-globin expression without effecting cell proliferation [86]. Erythroid precursor cells from β-thalassemia patients may differ in their response to the same inducer. Combined use of HbF inducers exhibiting different mechanism of action might improve the results.

- Cytotoxic agents

Observations that the use of cytotoxic agents helps to reactivate HbF synthesis during recovery from bone marrow suppression haves given hope on the possible use of cytotoxic agents for the treatment of serious haemoglobin disorders. Several cytotoxic agents that modify the pattern of erythropoiesis, increasing the expression of fetal (γ)-globin genes, have been explored over the past two decades [87–88]. Cytotoxic compounds put an end to actively cycling progenitors and control cellular growth to trigger rapid erythroid-regeneration and the formation of F cells. Cytotoxic drugs such as vinblastine [89], busulfan [90], cytosine arabinoside [91], and hydroxyurea [92–94] are known inducers of HbF in humans through this mechanism. Rapid regeneration of erythroid cells allows progenitors with an active eHbF program to be selectively recruited for maturation.

- Short-chain fatty acids (SCFAs)

Two different studies have reported that the infants of diabetic mothers have a delayed fetal-to-adult hemoglobin switch [95–96], while the exact underlying mechanisms are not clear, it is known that hydroxybutyrate is elevated during pregnancy induced diabeties in women. Perrine and colleagues observed that butyrate or other similar short chain fatty acids may act as effective inducers of fetal hemoglobin in sheep [95].

In support to the above observation, many other studies have shown that Sodium phenylbutyrate and arginine butyrate enhance both fetal and total hemoglobin levels in patients with sickle cell disease and beta thalassemia [97–106]. However, these SCFA derivatives also inhibit histone deacetylases, which generally inhibit erythroid cell proliferation. Therefore, these are not preferred as oral therapeutics that induce fetal (γ)-globin gene expression in beta hemoglobinopathies.

Loss of response to butyrates in long term therapy is observed which may be the result of antiproliferative effects on the bone marrow [107]. Two cohort studies have examined the efficacy of the oral butyrate derivative isobutyramide to induce HbF. In some studies no change in globin chain imbalance or in markers of ineffective erythropoiesis was noted, while in other study a drop in plasma free hemoglobin was observed. It has been suggested that the loss of response to long term butyrate therapy in patients with β-thalassemia may be because of the effects of these agents on other globin chains. It was shown that butyrate increases α-globin expression in progenitor-derived erythroid cells from patients with β-thalassemia. Thus, suggesting that the complementary effects of the butyrate-induced γ-globin expression on α/

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β-chain imbalance in β-thalassemia may be decreased as a result of the associated increase in α-globin expression [108].

Using insilico modeling, Perrine and associates have tested several SCFA derivatives to deduce the functional group(s) required for γ-globin reactivation [109]. Compounds such as phenoxyacetic acid, and 2, 2 dimethylbutyric acid and α-methyl hydrocinnamic acid, induce Stat-5 cell signaling and the growth-related immediate early genes c-myc and cmyb to achieve HbF induction independent of HDAC inhibition; butyrate has similar effects [101,110].

Short chain fatty acids, like Phenylbutyrate and Valproic acid, are also known to induce HbF levels in vivo [111–112]. Animal model studies have shown that propionate, phenyl acetic, and phenylalkyl acids induces HbF in baboons, transgenic mice, cultures, and in primates [103, 113–115].

- DNA methyl transferase (DNMT) inhibitors

DNA methylation is an epigenetic method of modulating gene expression carried out by enzyme DNA methyltransferases (DNMT) in both eukaryotes and prokaryotes [116]. These enzymes catalyze the addition of a methyl group from a donor S-adenosylmethionine to the 5′ position of cytosine, predominantly within the CpG dinucleotide [117–118].

CpG methylation in the promoter of adult erythroid cells is known to silence γ-globin gene expression [119]. The cytosine analogues such as, 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine), are known to inhibit DNMTs and can be used to reactivate γ-gene expression [120–122].

The γ-globin genes are structurally and functionally similar in baboons and humans and are developmentally silenced by methylation of CpG residues [123]. 5-Azacytidine was shown to increase HbF levels in baboons and sickle cell patients, but concerns over the carcinogenic effect of 5-azacytidine hampered its development for human treatment [124–126].

Another DNA methylation inhibitor adenosine-2 3-dialdehyde (Adox) is known to inhibit both DNA methylation and protein methylation including histone tail methylation. It is known to inhibit adenosylhomocysteine hydrolase activity, thereby indirectly inhibiting methyltransferases that catalyze adenosylmethionine to adenosylhomocysteine [127]. In order to test the effect of Adox to induce γ-globin, He et al. performed a series of studies. Firstly, they treated K562 cells and showed a dose response effect on activation. Next, they performed a time-course analysis of γ-globin induction by Adox. They observed that from day two, γ-globin expression was readily detected, but after day six, induction stopped suggesting that Adox could induce γ-globin very quick and it could also be metabolized during cell proliferation. Adox also induced a dose-dependent inhibition of in vitro proliferation of K562 cells. They also performed bisulfite DNA sequencing experiments on globin genes. Using decitabine as a positive control, they found that Adox significantly reduced DNA methylation. Together, these results suggested that Adox was a potent inducer of γ-globin expression in K562 cells [128].

In their previous study, the levels of histone mark H4R3me2s on the γ-globin promoter triggered by PRMT5 were significantly reduced in Adox-treated and decitabine-treated cells compared to untreated cells. PRMT5 inhibition by Adox treatment followed a dose response

that occurred over the same drug concentration range as γ-globin induction, indicating specificity of Adox for γ-globin gene.

However, DNA hypomethylation by Adox treatment may not be a major cause of the reactivation γ-globin expression in human β-thalassemia cells. But we can't exclude the possibility that by Adox treatment, different erythroid specific transcription factors play roles upon γ-globin induction. Alternatively, histone modification or repressor complex (e.g., NURD complex) reconstitution might also contribute to the γ-globin gene reactivation [129–131].

- Histone Deacetylase (HDAC) inhibitors

Histone deacetylases (HDACs) remove the acetyl group from histones resulting in the formation of tightly supercoiled, transcriptionally silent, "heterochromatin" structure that exerts antagonistic epigenetic controls on gene expression through chromatin re-modeling resulting in gene activation or repression [132–136]. HDACs are also known to deacetylase non-histone proteins, including many regulatory genes and transcriptional factors involved in cell signalling path and metabolism [137–138]. These observations suggest that HDACs plays an important role as co-repressors and co-activators of gene expression [139].

Till now, 18 mammalian HDAC genes have been identified that have been classified into four groups based on sequence homologies and catalytic mechanism [138, 140]. Class I includes HDACs 1-3, 8 and are expressed ubiquitously and consist mainly of a deacetylase domain. Class II includes HDACs 4-7 and 9-10, and are highly expressed in muscle, brain, and T cells [141]. Class I, II, and IV HDACs are evolutionarily related Zn^{+}-dependent hydrolases whereas, Class III HDACs have homology with yeast HDAC, silent information regulator 2, and use nicotinamide adenine dinucleotide as a co-substrate [142]. They have an extended N-terminus, which serves as a target for post-translational modifications and protein-protein interactions, such as phosphorylation, important for their function and governing nuclear-cytoplasmic shuttling [143].

A study by Forsberg et al. have shown that some specific histone acetylation patterns play a role in β-globin switch in murines, suggesting that the HDACs might participate in a complex mechanism of γ-globin repression, hence can be used for pharmacological reactivation of γ-globin expression [85, 144].

Shalini et al. (2010) screened Class II HDAC family members. They choose this class because they are highly tissue-specific in expression and have nuclear localization and import signals required for cell proliferation and differentiation [145–147]. Histone deacetylase inhibitors, including sodium butyrate (NaB) and trichostatin A (TSA) and hemin are known to significantly alter the mRNA levels of HDAC7, -9, and -10 and histone deacetylase-related protein (HDRP) at the same time activating γ-globin expression, as the result of loss in HDAC activity at the promoter or can also be because of changes in the expression of other genes [147]. This was verified by studying siHDAC9 in K562 cells, a dose-dependent decrease in γ-globin gene expression was observed, where forced expression of HDAC9 reactivated the γ-globin expression [147]. This study suggests that HDAC9 might have a positive role in regulating γ-globin expression.

A rapid and efficient method for detecting HbF inducers, based on a recombinant DNA construct was developed by Skarpidi and colleagues [148]. In this method, the coding sequences of the firefly and renilla luciferase genes were substituted for the human γ-globin and β-globin genes, respectively. Many new HDAC inhibitors were identified in this system and were further confirmed in human erythroid progenitor cultures [148]. With the help of the same reporter system, Makala and Pace found a stable reporter system in human KU812 cells [149]. The chemical analogues of HDAC inhibitor FK228 were identified as novel HbF inducers in primary erythroid progenitors. Last two decades has seen tremendous growth to identify other agents, systems with the help of high throughput screening techniques. Using this approach, selective HDAC 1/2 inhibitors (e.g., ACY-957) which induced HbF were reported [150]. Studies with ACY-957 have demonstrated elevated HbF expression in human erythroid progenitors [151].

Inducer	Mechanism of action	References (a)
Peptiole nucleic acids (PNAs)	Artificial promoters	Wang et al. (1999)
Cisplatin and analogues	DNA-binding drug	Bianchi et al. (2000)
Triple-helix oligodeoxynucleotides	Activation of γ-globin gene promote	Xu et al. (2000)
Trichostatin	HDAC inhibitors	Marianna et al. (2001)
Tallimustine and analogues	DNA-binding drug	Bianchi et al. (2001)
Angelicin	DNA-binding drug	Lampronti et al. (2003)
Mithramycin	DNA-binding drug	Fibach et al. (2003)
Citarabine	Hypomethylation of DNA	Sauthararajah et al, 2003
Hydroxyurea	Inhibition of DNA synthesis	Fibach et al. (2003)
Apicidin	HDAC inhibitors	Witt et al. (2003)
Rapamycin	FRAP-mTOR signal transduction	Mischiati et al. (2003) Fibach et al. (2006)
Scriptaid	HDAC inhibitors	Johnson et al. (2005)

Table 1. Inducers of Fetal Hemoglobin in Erythroid Precursor Cells from Human Donors

5.4. Other novel agents under development

Usually, developing a novel agent from bench to bedside takes more than 20 years on an average; thus, repurposing that FDA approved agents is a strategy encouraged by drug companies and government funding agents to expand the agents available to treat β-hemoglobinopathies. One such agent is the FDA approved Tecfidera (dimethyl fumarate), used for the treatment of multiple sclerosis. This agent acts via mediating immunemodulatory actions [152] with limited side effects. Dimethyl fumarate activates NRF2 signaling involved in drug mediated HbF induction [153–154]. Researchers have reported that dimethyl fumarate induces γ-globin expression in KU812 and primary erythroid cells generated from sickle cell patients, thereby supporting repurposing of Tecfidera for SCD [155].

Another FDA approved agent pomalidomide is under development for SCD. This agent stimulates proliferation of erythroid progenitors and HbF induction [156]. In a pre-clinical study, pomalidomide was investigated in the SCD knockout transgenic mouse produced by Townes and colleagues and was shown to induce HbF similar to hydroxylurea without myelo suppression. This study led to a Phase 1 clinical trial completion [157–159].

Preclinical studies to support the repurpose of the monoamine oxidase inhibitor tranylcypromine for SCD have been completed. The nuclear receptors TR2 and TR4 repress γ-globin expression by association with the co-repressors DNMT1 and lysine-specific demethylase 1 (LSD1) [58–59]. LSD1 removes methyl groups from mono- and dimethyl histone H3 lysine 4 producing an activating epigenetic signature [160]. The role of LSD1 in globin gene regulation in human erythroid cells was examined by Shi and colleagues [161]. They demonstrated that LSD1 was inhibited by tranylcypromine which mediated and enhanced HbF expression. Subsequent pre-clinical studies using β-YAC transgenic mice treated with tranylcypromine produced HbF induction. This served the basis for a clinical trial to test the ability of this agent in order to induce HbF in β-hemoglobinopathy patients [16].

5.5. Clinical development of HbF inducer

Till date, the most successful HbF inducer in the pharmacological armamentarium of drugs is Hydroxurea (HU). The first description of HU treatment in patients with SCD dates back to the year 1984 [162]. Since then, HU was found to elevate HbF levels and decrease the clinical complications of SCD and finally in 1998 it became the only FDA approved drug for SCD. In a Multicenter Study of Hydroxyurea (MSH), adult subjects with SCD were randomized on HU therapy or placebo and individuals in the HU group had ~50% reduction in vaso-occlusive rates [163]. Patients with maximal change in HbF levels had the highest reduction in white blood cell counts, indicating a hematologic response was associated with the clinical response [164]. The MSH data also demonstrated that treatment with HU was not associated with significant toxicities and was effective at reducing healthcare costs associated with complications due to SCD [163, 165]. Long-term follow up of MSH patients up to 17.5 years reported that there was a reduction in mortality [166]. Similar to these results, it was observed in the 17-year open label Laikon Study of HU in Sickle Cell Syndromes [167]; the probability of 10-year survival was 86% and 65% for HU and non-HU patients, respectively [167].

5.6. HU treatment in children

The efficacy and safety in adult trials was proved, and therefore the investigations of HU were undertaken in children. A pilot study of HU (HUSOFT) was conducted in 28 children with SCD where HU therapy was found to be feasible, well tolerated, and efficacious in young children. In addition, based on liver, spleen scans, HU could possibly delay functional asplenia [168]. Long-term follow-up data of patients in the HUSOFT extension study showed a reduction in acute SCD complications, improved splenic function, and improved growth rates in children taking HU therapy [169]. Based on these findings, BABY-HUG, a Phase III randomized controlled trial, was conducted in children with SCD (mean age 13.6 months) [170]. The primary endpoints of improved splenic function and renal glomerular filtration rates were not

achieved. HU was effective at reducing the acute complications of SCD, such as pain episodes, dactylitis, acute chest syndrome, and red blood transfusions [170]. The researchers were interested to use HU to prevent the long-term organ damage of SCD or to avoid chronic transfusions for children with severe phenotypes. Evidence of organ protection from HU in the current literature led to investigations of HU in this setting [171–173]. The Stroke With Transfusions Changing to Hydroxyurea (SWiTCH) study was a Phase III multicentred randomized trial compared the HU or phlebotomy with transfusions and chelation for the prevention of secondary stroke and reduction of transfusion iron overload [174–175]. There were no subsequent strokes on the transfusions/chelation arm but (10%) on the HU/phlebotomy arm. No major difference in liver iron content was found between the groups; therefore, the study was closed early. The investigators confirmed that the transfusions and chelation remain the standard of management of children with SCD and stroke complicated by iron overload [174]. Moreover, the studies in children have yet to demonstrate a role of HU in the prevention or management of chronic complications.

5.7. Natural inducers

Recently, scientists have conducted many studies to identify the natural remedies that could possibly be applied for the treatment of β-thalassemia (Table 2) [176]. Several studies have found that the extracts from medicinal plants for biomedical purposes [177–186] including therapeutic strategies can be used for the treatment of a number of diseases.

In case of hemoglobinopathies, only a few examples are available. For instance, the extract of Aegle Marmelos containing bergatene was found to activate erythroid differentiation and HbF induction in human leukemic K562 cells [187–188]. Citropten and bergatene are the two active ingredients in bergamot juice. They are potent inducers of Erythroid cell differentiation, $^{G}\gamma$-globin gene expression and fetal hemoglobin synthesis in human erythroid cells. Thus, it is known as a possible therapeutic approach for both β-thalassemia and Sickle cell anemia [187].

Another example of HbF inducer from the natural world is Angelicin, which can be found in the fruit of *Angelica arcangelica*. There is evidence demonstrating that angelicin is a strong inducer of Erythroid cell differentiation, improvement of the HbF synthesis, and γ-globin mRNA accumulation of human leukemia K562 cells [84, 179]. Red wine, particularly the skin of black grapes, contains resveratrol which mimics the HbF-inducing activity of HU. Its role in increasing the γ-globin mRNA in human erythroid precursors has been confirmed [84]. Since β-thalassemia cells present a high level of oxidative stress resulting in short time survival of erythroid cells in β-thalassemia patients, resveratrol which exhibits both antioxidant activity and HbF inducing property can be a very good HbF inducer from the natural world [88].

Rapamycin, which is isolated from *Streptomyces hygroscopicus*, is a bacterial species found in soil of Easter Island has the capability of increasing HbF production in cultures of erythroid precursors from β-thalassemia patients without any cytotoxicity or growth-inhibitory effect. Apart from Rapamycin, Mithramycin is a DNA-binding drug that is easily isolated from Streptomyces has the potential to induce γ-globin mRNA accumulation and HbF production in erythroid cells from healthy subjects as well as β-thalassemia patients [189].

Inducer	Source	Biological effects	References
Bergaptene	*A. marmelos*	Erythroid differentiation of K562 cells	Lampronti et al. (2003)
Angelicin	*A. arcangelica*	HbF production	Lampronti et al. (2003)
Rapamycin	*S. hygroscopicus*	HbF production	Mischiati et al. (2004)
Resveratrol	Redwine, grape skin and darakchasava	HbF production	Rodrigue et al. (2001)
Mithramycn	*Streptomyces* species	HbF production	Bianchi et al. (1999)
YiSui ShengXue Granule		Erythroid survival, proliferation, and terminaldifferentiation of K562 cells and HbF production	Zhang and Wu (2008)
Cucurbitacn D	Ethanol extract of Fructus trichosanthis, which is the fruit of Trichosanthes kirilowii MAXIM	HbF production and Erythroid differentiation of K562cells	Liu et al. (2010); Li et al. (2011)
Terminalia catappa distilled water active fraction (TCDWF)	*Terminalia catappa leaves*	Erythropoiesis, cell proliferation, and transcription	Aimola et al. (2014)

Table 2. Inducers of HbF from natural sources

6. Discussion and conclusion

The Human Genome Project has improved efforts of developing gene-based therapy for β-hemoglobinopathies alongside chemical inducers. GWAS have found certain major genetic modifiers of γ-globin, including -158 XmnI HBG2, HBS1-MYB and BCL11A accounting for ~50% of inherited HbF variance [190–191]. Orkin and colleagues advanced the field significantly by defining mechanisms through which BCL11A repressed γ-globin expression and holds the promise for the development of gene-based therapy in the future.

The occurrence of inherited mutations in KLF1 that produce HPFH suggested that this factor is a viable target for gene therapy and might be accomplished by RNAi technology to create a haploinsufficiency state. Promising molecular targets such as KLF1 and BCL11A for therapeutic efforts aimed at HbF induction have been identified: however, additional pre-clinical data are needed before manipulation of transcription factors can be translated into therapeutic options at the bedside.

Agents which improve HbF synthesis represent a rational approach for the treatment of β-thalassemia. For more than three decades, many chemical agents have been tested in tissue culture as HbF inducers, but few have crossed the path from clinical trials and have reached to the bedside. One major roadblock is the lack of suitable pre-clinical models to test agents in vivo. Baboon studies led to the clinical development of HU, butyrate, decitabine, and

HQK-1001 in SCD and β-thalassemia [93, 120–121, 124–125, 192–197]. With the establishment of SCD mouse models, newer agents such as pomalidomide have been progressed to clinical trials, however, the limited availability of the knockout SCD mouse model has affected progress in this area [155]. Townes and colleagues had designed a second knockin SCD mouse model with γ-globin and β-globin constructs [157]. However, HbF induction has not been effectively achieved with HU therapy (Pace, unpublished data). Therefore, the knock-in mouse may not be ideal for future pre-clinical drug screens. An alternative model, the β-YAC mouse was used to confirm in vivo HbF induction by 5-azacytidine, scriptaid, and transcyclomide showing the utility of this mouse for pre-clinical drug screening [161, 198–199]. Currently, very few agents are in clinical trials targeting HbF induction and drugs aimed at other complications of SCD, such as nitric oxide deficiency, endothelium antagonist, anti-platelet agents, anti-sickle agents, and so forth are still under development (clinicaltrials.gov). These can be combined with HU or other HbF inducers to produce an additive or synergistic clinic benefit [155].

Despite the success of HU in clinical trials conducted on adults and children and its proven safety and efficacy, it remains underutilized in SCD [163–164, 166, 168–170, 173, 200–201]. Possible causes include: (1) limited access to comprehensive sickle cell medical care; 2) lack of coordination between subspecialists and community based clinicians; 3) concern over potential genotoxicity; and 4) lack of patient adherence with the medication regimen. Future studies are needed to address early initiation of HU, the role of HU therapy in prevention of chronic complications, and improved methods for HU therapy delivery to all patients with SCD. There remains a question about the optimal age to initiate HU therapy. The Baby HUG trial established safety in young children, but additional clinical safety data are needed for children <6 months at the highest risk of infection and spleen and kidney dysfunction. Whether the early HU therapy administration will prevent the γ- to β-globin switch has not been demonstrated.

Curative therapy for SCD and β-thalassemia includes hematopoietic stem cell transplantation, however, this option is limited by the availability of suitable donors in <20% of children with SCD [202–203]. Ongoing clinical trials exploring alternative approaches such as matched unrelated donors and the development of new regimens using haplo-identical donors in the future will increase the transplant option for the majority of SCD patients [204]. Another approach to cure β-hemoglobinopathies is efforts to develop gene therapy, e.g., recently there was a successful treatment of two β-thalassemia patients with a modified β-globin lentivirus based vector. This progress holds promise for SCD patients [205–206].

Landmark studies have developed an excitement that fully differentiated somatic cells can be reprogrammed to make induced pluripotent stem cells [207]. Subsequent studies demonstrated rectification of a mouse model of SCD using this innovative approach, therefore opened the way to the use of these cells to cure β-hemoglobinopathies beside few limitations [208]. One limitation was the inability to restore all hematopoietic lineages with induced pluripotent stem cells which precludes using human therapy [209]. Therefore, until transplantation and gene therapy is more widely available, chemical inducers of HbF remain the most effective way to treat β-thalassemia and SCD.

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