

Chapter 2

Genetic Basis and Genetic Modifiers of β -Thalassemia and Sickle Cell Disease

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Abstract β -thalassemia and sickle cell disease (SCD) are prototypical Mendelian single gene disorders, both caused by mutations affecting the adult β -globin gene. Despite the apparent genetic simplicity, both disorders display a remarkable spectrum of phenotypic severity and share two major genetic modifiers— α -globin genotype and innate ability to produce fetal hemoglobin (HbF, $\alpha_2\gamma_2$).

This article provides an overview of the genetic basis for SCD and β -thalassemia, and genetic modifiers identified through phenotype correlation studies. Identification of the genetic variants modifying HbF production in combination with α -globin genotype provide some prediction of disease severity for β -thalassemia and SCD but generation of a personalized genetic risk score to inform prognosis and guide management requires a larger panel of genetic modifiers yet to be discovered.

Nonetheless, genetic studies have been successful in characterizing some of the key variants and pathways involved in HbF regulation, providing new therapeutic targets for HbF reactivation.

Keywords Sickle cell disease • β -thalassemia • Genotype/phenotype correlation • Fetal hemoglobin • Genetic prediction

Introduction

The inherited disorders of hemoglobin (Hb) production are the most common human monogenic disorders, among which those affecting the adult β globin gene (*HBB*)— β -thalassemia and sickle cell disease (SCD)—are the most clinically significant [1, 2]. β -thalassemia is caused by a spectrum of mutations that results in a quantitative reduction of β globin chains that are structurally normal [3], while SCD is caused by a single nucleotide substitution (GAG to GTG) in the sixth codon

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of *HBB* gene, substituting valine for glutamic acid in adult β globin ($\beta\text{Glu6Val}$), resulting in an abnormal Hb variant, HbS ($\alpha_2\beta_2^S$) [1, 4]. This change allows HbS to polymerize when deoxygenated, a primary event indispensable in the molecular pathogenesis of SCD.

Both β -thalassemia and SCD occurs widely in a broad belt of tropical and subtropical regions including the Mediterranean, parts of North and sub-Saharan Africa, the Middle East, Indian subcontinent and Southeast Asia, with some variations in frequencies in the two diseases. For example, SCD is predominant in sub-Saharan Africa but it is also prevalent in small pockets in the Mediterranean region, Middle East and the Indian sub-continent. It appears that heterozygotes for both thalassemia [5] and the β^S gene [6] are protected from the severe effects of falciparum malaria, and natural selection has increased and maintained their gene frequencies in these malarious regions. In these prevalent regions, gene frequencies for β -thalassemia range between 2 and 30%; in sub-Saharan Africa, the β^S gene frequency is uneven but rarely reaches 20% [7]. However, owing to population movements in recent years, both β -thalassemia and SCD are no longer confined to these high-incidence regions, but have become an important part of clinical practice posing an important public health problem in many countries, including North America and Europe [1].

The β -Globin Gene (*HBB*) and Normal Expression

Beta-globin is encoded by a structural gene found in a cluster with the other β -like genes on chromosome 11 (11p 15.15) [8]. The cluster contains five functional genes, ϵ (*HBE*), Γ (*HBG2*), Δ (*HBG1*), δ (*HBD*), and β (*HBB*), which are arranged along the chromosome in the order of their developmental expression to produce different Hb tetramers: embryonic (Hb Gower-1 ($\zeta_2\epsilon_2$), Hb Gower-2 ($\alpha_2\epsilon_2$), and Hb Portland ($\zeta_2\beta_2$)), fetal ($\alpha_2\gamma_2$), and adult (HbA, $\alpha_2\beta_2$ and HbA₂, $\alpha_2\delta_2$) [8]. Expression of the globin genes is dependent on local promoter sequences as well as the upstream β -globin locus control region (β -LCR) which consists of five Dnase 1 hypersensitive (HS) sites (designated HS1 to HS5) distributed between 6 and 20 kb 5' of *HBE* gene [9–11]. There is one HS site at approximately 20 kb downstream of *HBB* gene. All these regulatory regions bind a number of key erythroid-specific transcription factors, notably GATA-1, GATA-2, NF-E2, KLF1 (also known as EKLF), and SCL as well as various co-factors (e.g., FOG, p300), and factors that are more ubiquitous in their tissue distribution, such as Sp1 [8, 12, 13].

The β -like globin genes are each expressed at distinct stages of development through a process referred to as hemoglobin switching (embryonic \rightarrow fetal \rightarrow adult). Transcription of *HBE* in the embryonic yolk sac during the initial period of pregnancy switches during the second month of gestation to transcription of the γ -globin genes, and then around the time of birth, to that of the adult β -globin gene. At 6 months after birth, fetal Hb (HbF, $\alpha_2\gamma_2$) which comprises less than 5% of the total Hb, continues to fall reaching the adult level of <1% at 2 years of age, when

adult Hb becomes the major Hb [8]. It is at this stage that mutations affecting the adult *HBB* gene, i.e. β -thalassemia and SCD become manifested [14–16].

The tissue- and developmental-specific expression of the individual globin genes, i.e. hemoglobin switching, relies on a timely and direct physical interactions between the globin promoters and the β -LCR, the interaction being mediated through binding of erythroid-specific and ubiquitous transcription factors [17]. Tissue-specific expression may be explained by the presence of binding sites for the erythroid-specific transcription factors [12]. The binding of hemopoietic-specific factors activates the LCR, which renders the entire β -globin gene cluster transcriptionally active. Transcription factors which bind to enhancer and local promoter sequences within each gene, work in tandem to regulate the expression of the individual genes in the clusters. Some of the transcription factors are developmental stage specific and may be involved in the (still poorly understood) differential expression of embryonic, fetal and adult globin genes. A dual mechanism has been proposed for the developmental expression: (a) gene competition for the upstream β -LCR, conferring advantage for the gene closest to the LCR [18], and (b) autonomous silencing (transcriptional repression) of the preceding gene [19, 20]. The ability to compete for the β -LCR and autonomous silencing depends on the change in the transcriptional factor environment—in the abundance and repertoire of various transcription factors—favouring promoter-LCR interaction. While the ϵ and γ -globin genes are autonomously silenced at the appropriate developmental stage, expression of the adult β -globin gene depends on lack of competition from the upstream γ -gene for the LCR sequences. Concordant with this mechanism, when the γ -gene is upregulated by point mutations in their promoter causing a non-deletion hereditary persistence of fetal hemoglobin (HPFH), expression of the downstream *cis* β -gene is downregulated [21]. Further, mutations which affect the β globin promoter, which removes competition for the β -LCR, are associated with higher than expected increases in γ (HbF) and δ (HbA₂, $\alpha_2\delta_2$) expression [22–24]. A thorough understanding of the switch from fetal to adult Hb expression may provide insights on strategies of delaying the switch to allow persistent expression of the fetal globin genes for treating both SCD and β -thalassemia.

So far, the best-defined example of a developmental stage-specific regulatory factor is the erythroid Krüppel-like factor (EKLF, also known as KLF1) without which the β genes cannot be fully activated in the definitive cells [25–27]. Not only is KLF1 expression restricted mainly to erythroid cells [28] but it is also a highly promoter-specific activator, binding with high affinity to the β -globin CACCC box [24]. Its greater affinity for the β -globin than the γ -globin promoter accelerates the silencing of the γ -genes [29]. A genetic network regulating the switch from γ - to β -globin expression has emerged involving the interaction of KLF1, BCL11A and MYB with each other, and other transcription factors (e.g., GATA-1) and co-repressor complexes that involve chromatin modeling and epigenetic modifiers [30, 31]. BCL11A, previously known as an oncogene involved in leukemogenesis, was ‘discovered’ as an important genetic locus regulating HbF through genome-wide association studies (GWAS) [32, 33]. Downstream functional studies in cell lines, primary human erythroid cells and transgenic mice, have shown that BCL11A

is a repressor of γ -globin expression [34–36]. KLF1 is a direct activator of *BCL11A* [37, 38]. KLF1 is key in the switch from γ -globin to β -globin expression; it not only activates the β -globin gene directly, providing a competitive edge, but also silences the γ -globin genes indirectly via activation of *BCL11A*. KLF1 may also play a role in the silencing of the embryonic globin genes. KLF1 has emerged as a major erythroid transcription factor with pleiotropic roles underlying many of the previously uncharacterized anemias (e.g., congenital dyserythropoietic anemia type IV) [39–41]. KLF1 variants have also been associated with variable increases in HbF and HbA₂ levels, as a primary phenotype [39–41]. A recent case report indicated that complete absence of KLF1 causes hydrops fetalis and nonspherocytic hemolytic anemia [42].

Genetics of β -Thalassemia

Almost 300 β -thalassemia alleles have now been described (<http://globin.cse.psu.edu>) but only about forty account for 90% or more of the β -thalassemias worldwide. This is because in the areas where β -thalassemia is prevalent, only a few mutations are common, possibly reflecting local selection due to malaria. Each of these populations thus has its own spectrum of β -thalassemia alleles.

Downregulation of the β -globin gene can be caused by a whole spectrum of molecular lesions ranging from point changes to small deletions limited to the β -gene or extensive deletions of the whole β globin cluster or β LCR (Fig. 2.1) [3]. Deletions causing β -thalassemia, however, are rare; the vast majority (~250 of the 300) of mutations causing β -thalassemia are non-deletional.

Functionally, the β -thalassemia alleles can be considered as β^0 where no β -globin is produced, or β^+ in which some β -globin is produced, but less than normal. A range of severity is encountered within the β^+ thalassemia group; the less severe forms are sometimes designated β^{++} to reflect the minimal deficit in β chain production. Some β^{++} alleles are so mild that they are ‘silent’; carriers do not display any evident hematological phenotypes; their red cell indices and HbA₂ levels are within normal limits, the only abnormality being an imbalanced α :non- α chain synthesis [43]. These β -thalassemia alleles have usually been ‘discovered’ in individuals with thalassemia intermedia who have inherited a silent β -thalassemia allele in compound heterozygosity with a severe β -thalassemia allele. In this case, one parent has typical β -thalassemia trait, and the other (with the β^{++} thalassemia) is apparently normal.

Non-deletion β -Thalassemia

These non-deletional mutations, i.e. single base substitutions, small insertions or deletions of one to a few bases are located within the gene or its immediate flanking sequences. They downregulate the β -globin gene via almost every known stage of

Mutations downregulating β globin gene

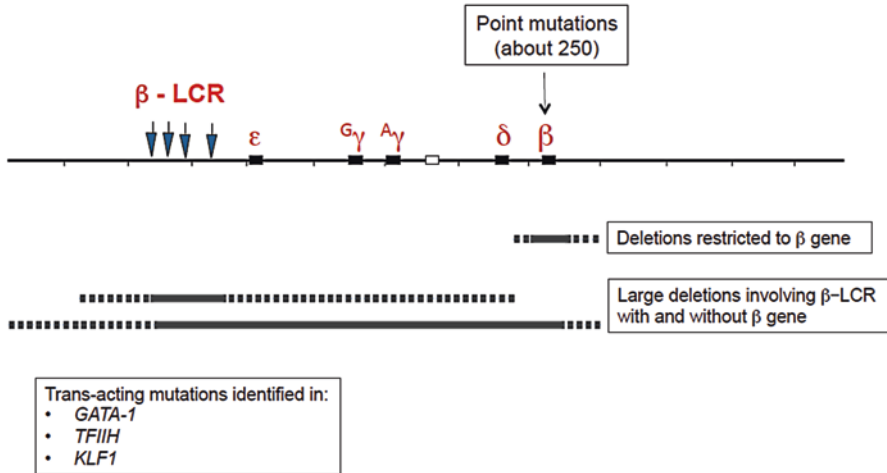


Fig. 2.1 Mutations causing β -thalassemia. A summary of the mechanisms downregulating β -globin gene expression. The upper panel depicts the β -globin gene (*HBB*) cluster with the upstream β -locus control region (β LCR). The vast majority are point mutations affecting the structural *HBB* gene. Deletions downregulating *HBB* are rare and are either restricted to the gene or extensive, involving the β LCR with or without *HBB*. *Dashed lines* represent variation in the amounts of flanking DNA removed by the deletions

gene expression, from transcription to RNA processing and translation of β -globin mRNA. Approximately half of the non-deletional mutations completely inactivate the β -gene with no β globin production resulting in β^0 thalassemia.

Transcriptional Mutations

Transcriptional mutants involve the conserved DNA sequences that form the β -globin promoter (from 100 bp upstream to the site of the initiation of transcription, including the functionally important CACCC, CCAAT and ATAA boxes) and the stretch of 50 nucleotides in the 5'UTR. Generally, these transcriptional mutants result in a mild to minimal reduction of β globin output i.e. β^+ or β^{++} thalassemia alleles, and occasionally they are 'silent'. A silent β -thalassemia allele which has been observed fairly frequently in the Mediterranean region is the -101 C \rightarrow T mutation where it interacts with a variety of more severe β -thalassemia mutations to produce milder forms of β -thalassemia [44]. Other 'silent' mutations include those in the 5' UTR; the extremely mild phenotype is exemplified in a homozygote

for the +1 A→C mutation who has the hematologic values of a thalassemia carrier, heterozygotes are ‘silent’ [45].

Within this group of transcriptional mutants, ethnic variation in phenotype has been observed. Black individuals homozygous for the -29 A→G mutation have an extremely mild disease [46], while a Chinese individual homozygous for the same mutation had severe anemia and was transfusion-dependent [47]. The cause of this striking difference in phenotype is not known but likely to be related to the different chromosomal backgrounds on which the apparently identical mutations have arisen. One difference is the C-T polymorphism at position -158 upstream of the γ globin gene (*XmnI*- γ site) present in the β chromosome carrying the -29 A→G mutation in Blacks but absent in that of the Chinese. The *XmnI*- γ site, considered to be a quantitative trait locus for HbF, is associated with increased HbF production under conditions of erythropoietic stress (see later on ‘Update on the genetic control on HbF’).

Mutations Affecting RNA Processing

A wide variety of mutations interfere with processing of the primary mRNA transcript. Those that affect the invariant dinucleotide GT or AG sequences at exon-intron splice junctions prevent normal splicing altogether, and cause β^0 thalassemia. Mutations involving the consensus sequences adjacent to the GT or AG dinucleotides, allow normal splicing to varying degrees and produce a β -thalassemia phenotype that ranges from mild to severe. For example, mutations at position 5 IVS1 G→C, T or A, considerably reduce splicing at the mutated donor site compared with the normal β allele [21]. On the other hand, the substitution of C for T in the adjacent nucleotide, intron 1 position 6, only mildly affects normal RNA splicing. Although the IVS1-6 T-C mutation is generally associated with milder β -thalassemia, studies have shown differential severities for apparently identical mutations; again this is presumably related to the chromosomal background on which the mutations have arisen [48].

Both exons and introns also contain ‘cryptic’ splice sites which are sequences very similar to the consensus sequence for a splice site but are not normally used. Mutations can occur in these sites creating a sequence that resembles more closely the normal splice site, such that during RNA processing the newly created site is utilized preferentially, leading to aberrant splicing; incorrectly spliced mRNA is not functional because spliced intronic sequences generate a frameshift and a premature termination code. Such mutations have been identified in both introns 1 and 2, and exon 1 of *HBB* gene. The associated phenotype may be either β^+ or β^0 thalassemia, depending on the proportion of normal and abnormal mRNA species generated. One such mutation is the (GAG → AAG) mutation in codon 26 in exon 1 [49], that results in the HbE variant. The single base substitution leads to a minor use of the alternative pathway; as the major β mRNA that codes for the variant is normally spliced, HbE has a mild β^+ thalassemia phenotype. Clinical phenotypes of

compound HbE/ β -thalassemia heterozygotes resemble those of homozygous β -thalassemia ranging from severe anemia and transfusion-dependency to non-transfusion dependent states (i.e. thalassemia intermedia) depending on the non-HbE β -thalassemia allele and other genetic factors [50].

Other RNA processing mutants affect the polyadenylation signal (AATAA) and the 3' UTR. These are generally mild β^+ thalassemia alleles [3].

Translational Mutations

About half of the β thalassemia alleles completely inactivate the gene mostly by generating premature stop codons (PTCs), either by single base substitution to a nonsense codon, or through a frameshift mutation. As part of the surveillance mechanism that is active in quality control of the processed mRNA, mRNA harboring a PTC are destroyed and not transported to the cytoplasm in a phenomenon called (nonsense mediated RNA decay or NMD) to prevent the accumulation of mutant mRNAs coding for truncated peptides [51]. However, some in-phase PTCs that occur later in the β sequence, in 3' half of exon 2 and in exon 3, escape NMD and are associated with substantial amounts of mutant β -mRNA leading to a synthesis of β chain variants that are highly unstable and non-functional with a dominant negative effect (see Dominantly inherited β -thalassemia) [52]. Other mutations of RNA translation involve the initiation (ATG) codon. Nine of these have been described; apart from an insertion of 45 bp all are single base substitutions and again they result in β^0 thalassemia [21].

Deletions Causing β -Thalassemia

β -thalassemia is rarely caused by deletions, 15 restricted to the *HBB* gene itself have been described, of which two remove the 3' end of the gene but leave the 5' end intact [3]. The 0.6 kb deletion at the 3' end of the β -gene is relatively common, but restricted to the Sind populations of India and Pakistan where it accounts for about one-third of the β -thalassemia alleles [53]. The other deletion which removes 7.7 kb 3' from the second intron of *HBB*, was described in compound heterozygosity with β^S gene in a woman with SCD from Cape Verde islands [54]. The other thirteen deletions differ widely in size (from 290 bp to >67 kb) but remove in common a region in the β promoter (from position -125 to +78 relative to the mRNA CAP site) which includes the CACCC, CCAAT, and TATA elements. They are extremely rare, but of particular clinical interest because they are associated with an unusually high levels of HbA₂ and HbF in heterozygotes. These deletions result in β^0 thalassemia, yet the increase in HbF is adequate to compensate for the complete absence of HbA in homozygotes for these deletions [55–57]. It has been proposed that the mechanism underlying the elevated levels of HbA₂ and HbF is related

to deletion of the β promoter removing competition for the upstream β -LCR and limiting transcription factors, resulting in an increased interaction of the LCR with the γ - and δ -genes in *cis*, thus enhancing their expression. This mechanism may also explain the unusually high HbA₂ levels that accompany the point mutations in the β promoter region [23].

Dominantly Inherited β -Thalassemia

In contrast to the common β -thalassemia alleles that are inherited typically as Mendelian recessives, some forms of β -thalassemia are dominantly inherited, in that inheritance of a single copy of the β -thalassemia allele results in clinical disease. Carriers have moderate to severe anemia, splenomegaly and the hallmarks of heterozygous β -thalassemia—elevated HbA₂ and imbalanced globin chain synthesis [58]. More than thirty dominantly inherited β -thalassemia alleles have now been described; they include a spectrum of molecular lesions—single base missense mutations and minor insertions / deletions that result in truncated or elongated β -globin variants with abnormal carboxy-terminal ends [59]. The underlying denominators of these variants are the production of highly unstable and non-functional β chain variants that are not able to form viable tetramers with α globin. These precipitate in the erythroid precursors and together with the redundant α chains, overload the proteolytic mechanism causing premature death of these cells, and accentuating the ineffective erythropoiesis. Unlike the recessive forms of β -thalassemia that are prevalent in the malarious regions, the dominantly inherited β -thalassemia variants are rare, and found in dispersed geographical regions where the gene frequency for β -thalassemia is very low. Furthermore, many of these variants are unique to the families described, and occur as de-novo mutations.

Unusual Causes of β -Thalassemia

The unusual causes of β -thalassemia are extremely rare and are mentioned here, not just for the sake of completeness but also to illustrate the numerous molecular mechanisms of downregulating the β -globin gene. Transposable elements may occasionally disrupt human genes and result in their activation. The insertion of such an element, a retrotransposon of the LI family into intron 2 of the β -globin gene has been reported to cause β^+ thalassemia [60].

Almost all variants downregulating *HBB* are physically linked to the gene and behave as alleles of the β -globin locus (i.e. they are *cis*-acting). Rarely, mutations in other genes distinct from the β -globin complex can downregulate β -globin expression. Such *trans*-acting mutations have been described affecting the XPD protein that is part of the general transcription factor TF1 IH [61], and the erythroid-specific GATA-1 [62]. Somatic deletion of the β globin gene contributed to thalassemia

intermedia in three unrelated families of French and Italian origins [63, 64]. The affected individuals with thalassemia intermedia were constitutionally heterozygous for β^0 thalassemia but subsequent investigations revealed a somatic deletion of chromosome 11p15, including the β globin gene complex, in *trans* to the mutation in a subpopulation of erythroid cells. This results in a somatic mosaic—10–20% of the cells were heterozygous with one normal copy of the β -globin gene, and the rest hemizygous, i.e. without any normal β -globin gene. The sum total of β -globin product is ~25% less than the asymptomatic β^0 carrier; these observations offer great promise for potential gene therapy as it shows that expression of a single β -gene in a proportion of red blood cells appears to be sufficient to produce a non-transfusion dependent state. Late presentation of β -thalassemia and transfusion dependency has been reported in a Chinese patient [65], and a Portuguese woman at the age of 15 years [66]. In both cases, the phenotype was caused by uniparental isodisomy of the paternal chromosome 11p15.5 that encompassed the β -thalassemia allele.

Genetics of Sickle Cell Disease

SCD describes a clinical syndrome caused by the presence of HbS (HbS G^{Glu6Val}) [4]. The main genotypes that contribute to SCD include homozygosity for the β^S allele (HbSS, specifically referred to as sickle cell anemia), followed by compound heterozygous states of HbS with HbC (HbSC disease). The third genotypic group includes compound heterozygotes of HbS with β -thalassemia, HbS β^+ thalassemia or HbS β^0 thalassemia depending on whether there is some, or no HbA, respectively. In African-descended populations, HbSS typically accounts for 65–70%, and HbSC 30–35% of the cases of SCD, with most of the remainder having HbS β -thalassemia (Table 2.1(A)). Other genotypes of SCD have been described, including compound heterozygotes of HbS with HbD, HbO-Arab, but these are rare (Table 2.1(B)). In all affected populations, the β^S gene is caused by the same molecular defect (β codon 6 GAG to GTG), found on a genetic background of four different β^S haplotypes: three distinct African haplotypes—Senegal, Benin and Central African Republic (or Bantu), and a fourth Indo-European β -haplotype (Arab-Indian) that is linked to the β^S gene in Saudi Arabian and Asian Indian patients [67]. The evidence suggests multiple independent origins of the β^S mutation although gene conversion on regionally specific β -haplotypes cannot be excluded.

Genetic Modifiers of β -Hemoglobinopathies

SCD and β -thalassemia are caused by mutations affecting a single gene, and yet, despite the apparent genetic simplicity, both disorders display remarkable diversity in the severity of their disease [4, 68, 69]. The mutations are detectable by DNA analysis, and although the information provides a basis for genetic counselling,

Table 2.1 Common genotypes of SCD

Genotype	Electrophoresis	Comments
<i>(A) Common genotypes of SCD</i>		
HbS/S (β Glu6Val/ β Glu6Val)	HbS 80–95%, No HbA, HbF 1–30%, HbA ₂ normal	Most severe and most common SCD ~65% in African patients
HbS/C (β Glu6Val/ β Glu6Lys)	HbS 45–50%, HbC 45–50% No HbA, HbF 1–6%,	Moderately severe. Frequency ~30% in African patients [152]
HbS/ β^0 thalassemia (β Glu6Val/ β^0 thalassemia)	HbS 80–95%, No HbA, HbA ₂ 4–6%,	Phenotype almost indistinguishable from HbSS (included as sickle cell anemia). Most prevalent in Mediterranean and Asian Indian patients [116]
HbS/ β^+ thalassemia (β Glu6Val/ β^+ thalassemia)	HbS 50–80%, HbA 5–30% HbA ₂ 4–6%, HbF 1–20%	Mild in African but severe in Mediterranean and Asian Indian patients [116]
HbS/E (β Glu6Val/ β Glu26Lys)	HbS 55–60%, HbE 30–35% HbA ₂ not distinguishable from HbE electrophoretically HbF 1–5%	Moderately severe, an increasingly common genotype [153]
HbS/HPFH	HbS 60–70%, HbF 25–35% No HbA if deletion HPFH Some HbA if non-deletion HPFH HbA ₂ 1.5–2.5% (decreased)	Very mild due to increased and pancellular HbF distribution [116]
<i>(B) Rarer genotypes of SCD</i>		
HbS/O Arab (β Glu6Val/ β Glu121Lys)		Severe SCD, relatively rare. Reported in north Africa, Middle East and the Balkans [116]
HbS/D Punjab (β Glu6Val/ β Glu121Gln)		Moderate to severe SCD, relatively rare. Predominant in north India but occurs worldwide [116]
HbS/C Harlem (β Glu6Val/ β Glu6Val, β Asp73Asn)		Very rare double mutation of β Asp73Asn on β^S allele; electrophoretically resembles HbS/C but severe SCD [154]

(continued)

Table 2.1 (continued)

Genotype	Electrophoresis	Comments
HbS Antilles/C (β Glu6Val, β Val23Ile/ β Glu6Lys)		Second mutation on β^S allele and results in severe disease when inherited with HbC; very rare [155]
HbS/Québec-CHORI (β Glu6Val/Ile, β Thr87Ile)		Resembles sickle trait with standard electrophoresis. Two cases described [156]
HbS/Hb Lepore (β Glu6Val/Hb Lepore) (fusion Hb)		Rare, first described in Greece but occurs worldwide. Hb Lepore migrates in same position as HbS on electrophoresis but can be separated by isoelectric focusing. Mild to severe SCD [157]
<i>(C) Unusual and very rare genotypes of SCD</i>		
Dominant form of SCD HbA/Hb Jamaica-Plain (β^A / β Glu6Val, β Leu68Phe)		Unique case of acquired new mutation (β Leu68Phe) on paternal β Glu6Val allele. Double mutant (Hb Jamaica-Plain) has markedly low oxygen affinity [158]
Dominant form of SCD HbA/S Oman β^A / β Glu6Val, β Glu121Lys)		Dominant form of SCD caused by 2nd mutation on β^S allele; very rare [159]
Uniparental disomy. Genetic heterozygote but functional hemizygote		Patient had paternal isodisomy of chromosome 11p that contained β^S allele resulting in subpopulation of erythroid progenitors homozygous for β Glu6Val [160]
Apparent heterozygosity for HbS β Glu6Val/Deletion of β LCR		Two cases reported. In both cases, HPLC showed no HbA and sequence analysis heterozygosity for $\beta 6$ gene [161]

predicting disease severity remains difficult. Identification of the genetic modifiers could provide more precise estimates of severity of disease, contributing to the ethos of precision medicine. Further, defining the molecular mechanisms linking the genetic factors could reveal new targets for therapeutic intervention.

Historically, the genetic modifiers in both β -thalassemia and SCD have been derived from an understanding of their pathophysiology, and subsequently validated by family and case control studies. Two important modifiers—co-inheritance of α

thalassemia and variants associated with increased synthesis of HbF in adults have emerged from such clinical genetic studies. The modifying effects of HbF and α -thalassemia on SCD and β -thalassemia at the molecular level could not be more different, and yet, elucidation of these genetic modifiers has not been too difficult as these loci have a major clinical effect and the genetic variants are common, and thus would contribute substantially to disease burden. However, these two modifiers do not explain all the clinical heterogeneity. Recent advances in technology and reducing costs have prompted numerous genome-wide association studies (GWAS) in an attempt to derive some of the genetic modifiers in such complex traits [70].

GWASs involve an unbiased scan of the whole human genome and, by design, are more likely to reveal unsuspected interactions [71]. A case in point is the application of GWAS in the highly successful discovery of *BCL11A* (an oncogene that hitherto, was not known to have a role in erythropoiesis) as a quantitative trait locus (QTL) controlling HbF [32].

Update on the Genetic Control of Fetal Hemoglobin (HbF)

The production of HbF is not completely switched off at birth; all adults continue to produce residual amounts of HbF, with over 20-fold variation [72]. Twin studies have shown that the common HbF variation in adults is predominantly genetically controlled; 89% of the quantitative variation is heritable but the genetic etiology is complex with no clear Mendelian inheritance patterns [73]. *Xmn1-HBG2* (*rs782144*) within the β -globin gene cluster on chromosome 11p, *HBS1L-MYB* intergenic region, (HMIP) on chromosome 6q23, and *BCL11A* on chromosome 2p16 are considered to be quantitative trait loci (QTLs) for the common HbF variation in adults [74–76].

Xmn1-HBG2 (*rs782144*) was the first known QTL for HbF and long implicated by clinical genetic studies [77]. SCD individuals in whom the β^S gene is on the Senegal or Arab-Indian β^S haplotype have the highest HbF levels and a mild clinical course, while SCD individuals with the β^S gene on a Bantu haplotype have the lowest HbF levels and the most severe clinical course [78, 79]. The differences in clinical severity was ascribed to the difference in HbF levels implicating the *Xmn1-HBG2* site which is linked to the Senegal and Arab-Indian β^S haplotype but not to the Bantu haplotype [77]. Recent high resolution genotyping, however, suggests that *rs782144* is not likely to be the variant itself, but in tight linkage disequilibrium with causal element(s) that remain to be discovered in the β -globin cluster.

Variants in the *HBB*, *HMIP* and *BCL11A* loci account for 10–50% of the variation in HbF levels in adults, healthy or with sickle cell anemia or β -thalassemia, depending on the population studied [32, 80–86]. The remaining variation (‘missing heritability’) is likely to be accounted for by many loci with relatively small effects, and/or rare variants with significant quantitative effects on γ -globin gene expression that are typically missed by GWAS population studies. An example of the latter is the *KLF1* gene [25, 40].

KLF1, discovered by Jim Bieker in 1993 [25] re-emerged as a key transcription factor controlling HbF through genetic studies in a Maltese family with β -thalassemia and HPPFH that segregated independently of the *HBB* locus [37]. Linkage studies identified a locus on chromosome 19p13 which encompassed *KLF1* and expression profiling of erythroid progenitor cells confirmed *KLF1* as the γ -globin gene modifier in this family. Family members with HPPFH were heterozygous for the nonsense K288X mutation in *KLF1* that disrupted the DNA-binding domain of KLF1, a key erythroid gene regulator. Numerous reports of different mutations in *KLF1* associated with increases in HbF soon followed (see review by Borg et al. 2011) [40]. The HbF increases occurred as a primary phenotype or in association with red blood cell disorders such as congenital dyserythropoietic anemia [39, 41], congenital nonspherocytic hemolytic anemia due to pyruvate kinase deficiency [87] and sickle cell anemia [40]. Several GWASs of HbF however, including ones in sickle cell anemia patients of African descent, failed to identify common variants [82, 86]. It would seem that *KLF1* does not play an important part in regulation of HbF in SCD. On the contrary, *KLF1* variants were over represented in Southern China where β -thalassemia is prevalent compared to North China. Further, *KLF1* variants were also over-represented in patients with thalassemia intermedia when compared to thalassemia major [88].

KLF1 is a direct activator of *BCL11A* (see below) and is also essential for the activation of *HBB* expression [38, 89]. Collectively, studies suggest that KLF1 is key in the switch from *HBG* to *HBB* expression; it not only activates *HBB* directly, providing a competitive edge, but also silences the gamma-globin genes indirectly via activation of *BCL11A*, and may play a role in the silencing of embryonic globin gene expression [87]. In the light of these findings, KLF1 has now emerged as a major erythroid transcription factor with pleiotropic roles underlying many of the previously uncharacterized anemias [30].

Functional studies in primary human erythroid progenitor cells and transgenic mice demonstrated that *BCL11A* acts as a repressor of gamma-globin gene expression that is effected by SNPs in intron 2 of this gene [34]. Fine-mapping demonstrated that these HbF-associated variants, in particular rs1427407, localized to an enhancer that is erythroid-specific and not functional in lymphoid cells [90]. *BCL11A* does not interact with the γ -globin promoter but occupies discrete regions in the *HBB* complex [91]. Functional studies in primary human erythroid progenitors and transgenic mice demonstrated that *BCL11A* represses γ -globin and the silencing effect involves re-configuration of the *HBB* locus through interaction with GATA-1 and SOX6 that binds the proximal γ -globin promoters [92, 93].

High resolution genetic mapping and resequencing refined the 6q QTL to a group of variants in tight linkage disequilibrium (LD) in a 24-kb block between the *HBS1L* and *MYB* gene, referred to as *HMIP-2* [94]. The causal single nucleotide polymorphisms (SNPs) are likely to reside in two clusters within the block, at -84 and -71 kb respectively, upstream of *MYB* [95, 96]. Functional studies in transgenic mice and primary human erythroid cells provide overwhelming evidence that the

SNPs at these two regions disrupt binding of key erythroid enhancers affecting long-range interactions with *MYB* and *MYB* expression, providing a functional explanation for the genetic association of the 6q *HBSIL-MYB* intergenic region with HbF and F cell levels [95, 97, 98].

A three-base pair (3-bp) deletion in *HMIP-2* is one functional element in the *MYB* enhancers accounting for increased HbF expression in individuals who have the sentinel SNP rs9399137 that was found to be common in European and Asian populations, although less frequent in African-derived populations [99]. The DNA fragment encompassing the 3-bp deletion had enhancer-like activity that was augmented by the introduction of the 3-bp deletion.

The *MYB* transcription factor is a key regulator of hematopoiesis and erythropoiesis, and modulates HbF expression via two mechanisms: (1) indirectly through alteration of the kinetics of erythroid differentiation: low *MYB* levels accelerate erythroid differentiation leading to release of early erythroid progenitor cells that are still synthesizing predominantly HbF [8], and (2) directly via activation of *KLF1* and other repressors (e.g., nuclear receptors TR2/TR4) of gamma-globin genes [98, 100, 101].

Modulation of *MYB* expression also provides a functional explanation for the pleiotropic effect of the *HMIP-2* SNPs with other erythroid traits such as red cell count, MCV, MCH, HbA₂ levels, and also with platelet and monocyte counts [102–105]. *MYB* expression is also reduced by GATA-1 [106], and miRNA-15a and -16-1 [107]. Elevated levels of the latter have been proposed as the mechanism for the elevated HbF levels in infants with trisomy 13. A delayed HbF to HbA switch, along with persistently elevated HbF levels, is one of the unique features in infants with trisomy 13 [108]. One study has provided compelling evidence that the elevated HbF levels relate to the increased expression of microRNAs 15a and 16-1 produced from the triplicated chromosome 13. The increased HbF effect is mediated, at least in part, through down-modulation of *MYB* via targeting of its 3' UTR by microRNAs 15a and 16-1 [107].

The *HBSIL-MYB* intergenic enhancers do not appear to affect expression of *HBSIL*, the other flanking gene [95]. Further, one study also excluded *HBSIL* as having a role in the regulation of HbF and erythropoiesis. In whole-exome sequencing of rare uncharacterized disorders, mutations in the *HBSIL* gene leading to a loss of function in the gene were identified in a female child [109]. The child had normal blood counts and normal HbF levels. Thus, *HMIP-2* is likely to affect HbF and hemopoietic traits via regulation of *MYB*. *MYB* was also causally implicated by fine-mapping which identified rare missense *MYB* variants associated with HbF production [110].

The emerging network of HbF regulation also includes SOX6, chromatin-modeling factor FOP and the NURD complex, the orphan nuclear receptors TR2 / TR4 (part of DRED) and the protein arginine methyltransferase PRMT5, involving DNA methylation and HDACs 1 and 2 epigenetic modifiers. Regulators of the key TFs, such as microRNA-15a and 16-1 in controlling *MYB*, could also have a potential role in regulating HbF levels [111].

Genetic Modifiers of Sickle Cell Disease

Two case reports [112, 113] and a pilot twin study [114] show that despite identical β and α globin genotypes and similarities in growth, hematological and biochemical parameters, the identical twins have quite different prevalence and severity of painful crises and some of the sickle complications. Although sample size of the twin study is small, nevertheless, it does suggest that environmental factors may be of greater importance in determining clinical expression and complications of sickle disease.

SCD should be considered as both a qualitative and quantitative genetic disorder. While presence of HbS is fundamental to the pathobiology, the likelihood of HbS polymerization and sickling is also highly dependent on the concentration of intra-erythrocytic HbS, as well as the presence of non-S Hb [115]. Thus, individuals with HbSS (SCD-SS) or HbS β^0 thalassemia, where the intra-cellular Hb is almost HbS tend to have the most severe disease followed by HbSC and HbS β^+ thalassemia (see Table 2.1).

HbA ($\alpha_2\beta_2$) or HbA₂ ($\alpha_2\delta_2$) do not participate in HbS polymerization, and since the β^+ thalassemia alleles in Africans are of the milder type with minimal deficit in β -globin production, HbS β^+ thalassemia in Africans tends to be very mild. In contrast, individuals with HbS β^+ thalassemia in the Mediterranean, have SCD almost as severe as that of HbSS [116]. Subjects with sickle cell trait (HbAS) with HbF of 35–40% do not normally suffer from symptoms of SCD. Under exceptional circumstances however, such as intense physical activity and dehydration, the consequent increased intracellular HbS concentration can induce vaso-occlusive pain [117].

Other genetic factors that influence the primary event of HbS polymerization include (HbF, $\alpha_2\gamma_2$) and the co-inheritance of α thalassemia (Table 2.2).

Impact of HbF in SCD

HbF inhibits the propensity for HbS polymerization: the hybrid tetramers ($\alpha_2\gamma\beta_s$), not only inhibits HbS polymerization, but intra-erythrocytic HbF presence also dilutes the concentration of HbS [118]. The clinical phenotype of SCD becomes evident within 6 months to 2 years of age as HbF levels decline.

Its impact at the primary level of disease pathology predicts HbF levels to have a global beneficial effect. Indeed, HbF levels are a major predictor of survival in SCD [119], and low levels of HbF have been associated with increased risk of brain infarcts in young children [120]. At the sub-phenotype level, apart from the clear benefit of high HbF levels with acute pain and leg ulceration, there are disparities and less conclusive evidence in its effects on complications such as stroke, renal impairment, retinopathy and priapism [121, 122]. The failure of HbF to uniformly modulate all complications of SCD may be related to the different pathophysiology of the different complications and perhaps also to the small sample sizes in genetic studies and even smaller numbers of end complications, and to ascertainment of phenotypes.

Table 2.2 Genetic modifiers of SCD

Modifier	Mechanism
<i>Primary at level of HbS polymerization and sickling</i>	
1. Sick genotype HbSS, HbSC, HbS β -thalassemia	Likelihood of HbS polymerization highly dependent on concentration of intracellular HbS
2. Innate ability to produce HbF ($\alpha_2\gamma_2$) through co-inheritance of HbF QTLs	HbF inhibits HbS polymerization and dilutes intracellular HbS concentration
3. Co-inheritance of α -thalassemia	α thalassemia reduces intracellular HbS concentration, increases Hb; clinical effects uneven, protective for some complications but predisposes to others
Complication	
<i>Secondary at level of sub-phenotypes and complications</i>	
1. <i>UGT1A1</i> promoter (TA) _n polymorphism	Serum bilirubin and propensity to gallstones
2. <i>MYH9</i> — <i>APOL1</i> locus	Proteinuria and sickle cell nephropathy
3. <i>ADYC9</i> , <i>ANXA2</i> , <i>TEK</i> and <i>TGFBR3</i>	Stroke risk, mechanisms unclear
4. <i>GOLGB1</i> and <i>ENPPI</i>	Stroke risk, mechanisms unclear
5. <i>TGFβ</i> / <i>SMAD</i> / <i>BMP</i> pathway	Multiple sub-phenotypes including osteonecrosis, acute chest syndrome, pulmonary hypertension, leg ulceration, renal impairment, infarction, priapism
6. <i>KLOTHO</i> (<i>KL</i>)	Priapism, mechanisms unclear
7. <i>NPRL3</i> on Chr16p	Reduces hemolysis, α -thalassemia effect suggested

Note: Most modifiers that affect SCD at the secondary level of sub-phenotypes and complications have not been replicated

Adapted from Thein, S.L. Genetic association studies in β -hemoglobinopathies. Hematology, ASH Education Program Book 2013; 354-61

HbF levels vary considerably from 1% to as high as 25% in individuals with SCA and behave as a quantitative genetic trait as in healthy individuals. As discussed earlier, part of this variation resides in regions linked to the *HBB* complex and is associated, at least in part, with the β^S haplotype (Senegal, Arab-Indian, Bantu and Benin) on which the β^S gene is found. However, a variance of HbF levels is encountered within each β^S haplotype, evidence for the importance of unlinked HbF QTLs such as HMIP-2 on 6q and *BCL11A* on 2p. Their effect on HbF levels varies with the frequency of the HbF boosting (minor) alleles on the three QTLs (β -globin cluster, HMIP-2 and *BCL11A*) in different population groups; in patients of African American patients with SCD, the three loci account for 16%-20% of the variation in HbF levels with a corresponding reduction in acute pain rate [80].

A recent GWAS in Tanzanian patients with SCD also confirmed association SNPs at *BCL11A* and HMIP-2 with HbF but no other HbF QTLs were ‘discovered’ [86]. Similarly, in patients from Cameroon, while these SNPs had a significant impact on HbF levels and rates of hospitalization, they explained less of the HbF variance than that observed in African American patients [123]. The *Xmn1-HBG* (*rs782144*) is virtually absent in Cameroon patients with SCD [123].

Impact of α -Thalassemia on SCD Phenotype

About one third of SCD patients of African descent have co-existing α -thalassemia due to the common deletional variant ($-\alpha^{3.7}$) [124]. The majority is heterozygous ($\alpha\alpha/\alpha-$) with 3–5% homozygous for the deletion ($\alpha-/ \alpha-$) [125]. Co-existing α -thalassemia reduces intracellular Hb concentration, decreases the propensity for HbS polymerization, and decrease hemolysis. While the co-existing α -thalassemias have a protective effect against complications associated with severe hemolysis such as priapism, leg ulceration and albuminuria, the increased hematocrit and blood viscosity may account for the increase in other complications associated with microvascular occlusion such as increased acute pain, acute chest syndrome, osteonecrosis and retinopathy [122].

Co-inheritance of α -thalassemia improved hematological indices and was associated with lower rates of hospitalization in Cameroon patients with SCA [126]. In the same study, Rumaney *et al* also showed that in Cameroon, the incidence of α -thalassemia trait in controls (HbAA) was significantly lower than that in patients with SCA (9.1 vs. 30.4%); the authors proposed that the difference in incidence could be explained by a protective effect of α thalassemia on overall survival in SCA patients. Co-inheritance of α -thalassemia was also associated with lower hospitalization rate in SCA patients.

Coexisting α -thalassemia reduces bilirubin with a quantitative effect that is independent to that of the *UGT1A1* promoter polymorphism [125]. In Jamaicans, the absence of α thalassemia and higher HbF levels predicts a benign disease, although a subsequent study reports that α -thalassemia did not promote survival in elderly Jamaicans with SCD-SS [127]. Coinheritance of α -thalassemia blunts the response to hydroxyurea therapy in SCD, that may be explained by its effect on HbF levels and MCV, two key parameters associated with hydroxyurea response [128]. It is quite likely that α -thalassemia carriers could have a poorer response to channel blockers that aim to reduce sickling through preservation of cell hydration [129].

Secondary Modifiers of Sub-phenotypes and Complications

Association studies of candidate genes implicated in the pathophysiology of vasculopathy, such as those encoding factors modifying inflammation, oxidant injury, nitric oxide biology, vaso-regulation and cell adhesion, have been used extensively to identify variants affecting sickle-related complications, such as stroke, priapism, leg ulcers, avascular necrosis, renal disease, acute chest syndrome, gallstones and susceptibility to infection (see following reviews [121, 122, 130]). The majority of these reported associations have not been replicated or validated, and are likely to be false positives. Of the numerous association studies reported, the most robust is the association between serum bilirubin levels and predisposition to gallstones with the 6/7 or 7/7 (TA) repeats in the *UGT1A1* promoter [125, 131]. The influence of *UGT1A1* polymorphism became more evident in patients while on

hydroxycarbamide therapy; children with 6/6 *UGT1A1* genotype achieved normal bilirubin levels while children with 6/7 or 7/7 *UGT1A1* genotypes did not [132].

Numerous genetic and clinical association studies on cerebrovascular complications in SCD have been carried out [133]. Of the 38 published SNPs associated with stroke, the protective effect of α thalassemia on stroke risk and SNPs in four genes (*ADYC9*, *ANXA2*, *TEK* and *TGFBR3*) could be replicated although only nominally significant association results were obtained [134]. More recently, GWAS in combination with whole exome sequencing have identified mutations in two genes—*GOLGB1* and *ENPPI*—associated with reduced stroke risk in pediatric patients but again, this needs validation in independent studies. In an attempt to overcome the small sample size in end-point complications, a study utilized a compound phenotype that included one or more sickle-related complications [135]. Patients with complications had a higher frequency of the platelet glycoprotein allele *HPA-5B*. In this small study, most of the complications were osteonecrosis and only four individuals had more than one complication [135]. As traditional methods are often inadequate in association studies of complex traits, methods of evaluating multi-locus data are promising alternatives. A GWAS was applied to SCD based on a disease severity score that was derived from a Bayesian network that integrates 25 different clinical and laboratory variables [136]. Several genes not known to be related in the pathogenesis of SCD were identified including *KCN6* (a potassium channel protein) and *TNK5* (gene encoding tankyrase-1, a possible telomere length regulator). However it is important to remember that results from such analytical techniques are dependent on the structure of a model which assumes causality and prior probabilities assigned to the different variables. Using a hemolytic score, GWAS identified a SNP (*rs7203560*) in *NPRL3* that was independently associated with hemolysis [137]. *rs7203560* is in perfect LD with SNPs within the α -globin gene regulatory elements (HS-48, HS-40 and HS-33), and also in LD with *ITFG3* that is associated with MCV and MCH in several GWASs of different population groups. It is proposed that *NPRL3* reduces hemolysis through an independent thalassaemic effect on the *HBA1/HBA2* genes.

Hydroxyurea remains a major treatment option for SCD [138, 139]; its main effect is mediated primarily through induction of HbF. Clinical and laboratory response to HU therapy however, is variable, a main determinant of response appears to be the baseline HbF levels. Numerous association studies on HbF response to hydroxycarbamide have been reported, of which the association with baseline HbF levels and *Xmn1-HBG2* seems to be the most robust [140]. Table 2.1 summarizes the genetic modifiers of SCD.

Genetic Modifiers of β -Thalassemia

The central mechanism underlying the pathophysiology of the β -thalassemias relates to the deleterious effects of the excess α -globin chain on erythroid survival and ineffective erythropoiesis [68]. Clinical studies have shown that disease severity correlates well with the degree of imbalance between α and β globin chains and the

size of the free α chain pool. Genetic modifiers can impact the phenotypic severity at the primary level by affecting the degree of globin chain imbalance, and at the secondary level by moderating complications of the disease related to the anemia or therapy e.g. iron chelation.

While the severity of β -thalassemia is primarily determined by the degree of β chain deficiency, for any given β -thalassemia allele the severity of the disease can be alleviated by co-inheritance of α -thalassemia or by co-inheritance of factors that increase γ -globin chain production and HbF levels. In the latter case, γ -globin chains combine with the excess α -globin to form HbF; cells that contain a relatively higher percentage of HbF are protected against the deleterious effect of alpha-globin chain precipitation and premature death, and have selective survival. Thus, all individuals with β -thalassemia have variable increases in HbF due to survival of these F cells.

Certain β -thalassemia mutations, notably those that involve small deletions or mutations of the promoter sequence of the *HBB* gene, are associated with much higher levels of HbF production than mutations affecting other regions of *HBB* (see deletions causing β -thalassemia) [3]. This might reflect the competition between the *HBG* and *HBB* promoters for interaction with the upstream β -LCR and limited transcription factors. Heterozygotes for such types of β -thalassemia mutations have unusually high HbA₂, and although the increases in HbF levels are variable, the increase in HbF production in homozygotes is adequate to compensate for the complete absence of HbA. HbF levels are normal or slightly elevated in β -thalassemia heterozygotes. Higher HbF (and HbA₂) levels are found with mutations that involve promoter of the *HBB* gene, but variations in HbF levels also reflect the genetic background of the individual (e.g. -29 promoter mutation in Blacks and Chinese). In homozygous β -thalassemia, the proportion of HbF ranges from 10% in those with the milder alleles to almost 100% in homozygotes or compound heterozygotes with β^0 thalassemia. In the most severe cases, the absolute amount of HbF is approximately 3–5 g/dL, produced as a result of extreme erythroid hyperplasia, selective survival of F cells, and some increase in *HBG* transcription. Non-transfusion dependent β^0 thalassemia intermedia with hemoglobin levels of 8–11 g/dL and 100% HbF has been observed. In some cases, the increase in HbF production reflects the type of β -thalassemia allele, but in others co-inheritance of QTLs associated with increased *HBG* expression might explain their more benign clinical features [114].

Effect of the Primary Modifiers: HbF Quantitative Trait Loci and α -Globin Genotype

While selection of F cells provides an explanation for the increases in HbF in β -thalassemia, the mechanism does not explain the wide variation in the amount produced. Much of this variability is genetically determined, in part from the co-inheritance of one or more of HbF-boosting alleles of the *Xmn1-HBG2*, *HMIP-2* and *BCL11A* HbF quantitative trait loci (QTLs) [33, 81, 84, 142, 143]. The *Xmn1-HBG2* QTL is a common sequence variation in all population groups, present at a frequency of approximately 0.35. Although increases in HbF and F cells associated

with *Xmn1-HBG2* are minimal or undetectable in healthy adults, clinical studies have shown that under conditions of stress erythropoiesis, as in homozygous β -thalassemia, the presence of *Xmn1-HBG2* leads to a much higher HbF response [141]. This could explain why the same mutation on different beta chromosomal backgrounds, some with and others without the *Xmn1-HBG2* variant, are associated with different clinical severity. High resolution genotyping studies suggest that *Xmn1-HBG2* may not be the causal element but in tight linkage disequilibrium to another, as yet undiscovered, variant(s) on chromosome 11p.

Together *Xmn1-HBG2*, *BCL11A*, and HMIP-2, and perhaps other loci, linked and unlinked to the *HBB* complex, constitute the loosely-defined entity of heterocellular HPFH. These HbF QTLs play an important role in fine-tuning γ -globin production in healthy adults and in response to the stress erythropoiesis of sickle cell anemia and β -thalassemia. The three QTLs are associated with HbF and severity of thalassemia in diverse population groups including Sardinian, French, Chinese, and Thai [33, 84, 143, 144]. More than 95% of Sardinian β -thalassemia patients are homozygous for the same codon 39 β^0 thalassemia mutation but have extremely variable clinical severity. Co-inheritance of variants in *BCL11A* and HMIP-2, and α -thalassemia accounts for 75% of the differences in disease severity [81]. In France, a combination of the beta thalassemia genotype, *Xmn1-HBG2* and SNPs in *BCL11A* and HMIP-2, can predict up to 80% of disease severity [84]. In a cohort of 316 β^0 thalassemia patients, delayed or absent transfusion requirements correlated with status of the three HbF QTLs and the α -globin genotype [145]. Using a combination of the HbF QTLs, the type of β -thalassemia mutations, and the α -globin genotype, a predictive score of severity has been proposed [146].

In many populations where β -thalassemia is prevalent, α -thalassemia also occurs at a high frequency and hence it is not uncommon to co-inherit both conditions [15]. Homozygotes or compound heterozygotes for β -thalassemia who co-inherit α -thalassemia will have less redundant α globin and tend to have a less severe anemia. The degree of amelioration depends on the severity of the β -thalassemia alleles and the number of functional α -globin genes. At one extreme, patients with homozygous β -thalassemia who have also co-inherited HbH (equivalent of only one functioning α -globin gene) have thalassemia intermedia [68].

In individuals with one β -thalassemia allele (heterozygotes), co-inheritance of α -thalassemia normalizes the hypochromia and microcytosis but the elevated HbA₂ remains unchanged. Increased α globin production through co-inheritance of extra α -globin genes (triplicated— $\alpha\alpha\alpha/\alpha\alpha$ or $\alpha\alpha\alpha/\alpha\alpha\alpha$, quadruplicated— $\alpha\alpha\alpha\alpha/\alpha\alpha$, or duplication of the whole α globin gene cluster— $\alpha\alpha/\alpha\alpha/\alpha\alpha$) with heterozygous β -thalassemia tips the globin chain imbalance further, converting a typically clinically asymptomatic state to thalassemia intermedia [14, 68, 147]. Again, the severity of anemia depends on the number of extra α globin genes and the severity of the β -thalassemia alleles.

At the primary level of chain imbalance, the proteolytic capacity of the erythroid precursors in catabolising the excess α globin has often been suggested, but this effect has been difficult to define. Alpha hemoglobin stabilizing protein, a molecular chaperone of α globin has also been suggested as another genetic modifier but its impact on disease severity has been inconclusive [148].

Secondary Modifiers of Complications of β -Thalassemia

These modifiers do not affect globin imbalance directly but might moderate the different complications of β -thalassemia that are directly related to the anemia, or to therapy such as iron overload [69, 149, 150]. They include genetic variants which affect bilirubin metabolism, iron metabolism, bone disease and cardiac complications. Jaundice and a predisposition to gallstones, a common complication of β -thalassemia, are associated with a polymorphic variant in the promoter of the *UGT1A1* gene. Individuals who are homozygous for 7 [TA]_ns, also referred to as Gilbert's syndrome, have higher levels of bilirubin and increased predisposition to gallstones, an observation that has been validated at all levels of β -thalassemia. Several genes involved in iron homeostasis have now been characterized, including those encoding HFE, (*HFE*), transferrin receptor 2 (*TFR2*), ferroportin (*FPN*), hepcidin (*HAMP*) and hemojuvelin (*HJV*) [151]. The H63D variant, a common polymorphism in the *HFE* gene, appears to have a modulating effect on iron absorption. β -thalassemia carriers who are homozygous for *HFE* H63D variant, have higher serum ferritin levels than carriers without the variant (see Table 2.3 for modifiers of β -thalassemia).

Table 2.3 Genetic modifiers of β -thalassemia

Modifier	Mechanism
<i>(A) Primary at level of α : non-α globin chain imbalance</i>	
1. β globin genotype (one or two, and severity of β -thalassemia alleles)	Directly affects output of β globin and chain imbalance
2. α globin genotype	
α thalassemia	Reduces chain imbalance and α globin excess
co-inheritance of extra α globin genes ($\alpha\alpha\alpha'$, $\alpha\alpha\alpha\alpha'$, or <i>HBA</i> cluster duplication)	Increases α globin excess and chain imbalance
3. Innate ability to increase HbF (co-inheritance of HbF QTLs, eg, SNPs in <i>BCL11A</i> , <i>HMIP</i> , <i>Xmn1-HBG2</i>)	Increased γ chains combine with excess α reducing chain imbalance
4. α hemoglobin stabilizing protein (<i>AHSP</i>)	Chaperones excess α globin (studies inconclusive)
<i>(B) Secondary at level of complications related to disease and therapy</i>	
Complication	Modifier
1. Serum bilirubin and propensity to gallstones	<i>UGT1A1</i> promoter (TA) _n polymorphisms
2. Iron loading	<i>HFE</i> - H63D variants increase GI absorption of iron
3. Osteopenia and osteoporosis	Variants in <i>VDR</i> , <i>COL1A1</i> , <i>COL1A2</i> , <i>TGFBI</i> , modify bone mass
4. Cardiac disease	Apolipoprotein (<i>APOE</i>) $\epsilon 4$ —risk factor for left ventricular heart failure
5. Cardiac iron loading	Glutathione-S-transferase M1—increased risk of cardiac iron in thalassemia major

Note: Adapted from Thein, S.L. Genetic association studies in β hemoglobinopathies. Hematology, ASH Education Program Book 2013; 354-61

The degree of iron loading, bilirubin levels and bone mass are quantitative traits with a genetic component; variants affect the genes that are involved in the regulation of these traits that contribute to the complications.

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