Genetic modifiers of β-thalassemia

R. Galanello

2nd Pediatric Clinic, Ospedale Regionale Microcitemie, ASL8 University of Cagliari, Cagliari, Italy

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A B S T R A C T

The clinical manifestations of β-thalassemia are extremely heterogeneous, ranging from severe transfusion-dependent anemia, to the asymptomatic carrier state. The remarkable phenotypic variability is primary due to variations in the different globin genes (primary genetic modifiers). The main pathophysiological determinant of the severity of β-thalassemia syndromes is the extent of α/non-α globin chain imbalance. Therefore, any factor capable of reducing the globin chain imbalance may have an ameliorating effect on the clinical picture. The most common mechanisms responsible of the amelioration of the phenotype are mild or silent β-thalassemia alleles, coinheritance of α-thalassemia, or of genetic determinants associated with increased γ globin chain production. Recently, major advances have been made in the discovery of critical modifier loci of γ globin production, such as HBS1L-MYB and especially BCL11A, a master regulator of fetal hemoglobin and hemoglobin switching. Polymorphisms of BCL11A, HBS1L- MYB, and γ-globin genes (XmnI G-γ) account for of the variability in the clinical phenotypes in β-thalassemia. In addition to the variability of the phenotype resulting from primary genetic modifiers, other genetic factors (secondary genetic modifiers), mapping outside the β and α globin cluster, may influence the disease complications. Among these, the best ones defined so far are those affecting bilirubin, iron, and bone metabolism. However, the new methods of DNA analysis (*i.e.*, GWAS and related methods) are expected to expand the number of genes or genetic variants involved in the phenotypic variability and in the response to treatment of β-thalassemia.

Introduction

β-thalassemia, one of the commonest monogenic disorders, are a group of hereditary blood disorders characterized by variably reduced (β^+) or absent (β°) synthesis of the β globin chains. More than 200 β-thalassemia defects have been so far identified (list available at http://globin.cse.psu.edu/glibin/Hbvar), but population studies indicate that 40 account for more than 90% of the β-thalassemias worldwide.[1] The large majority of mutations are single nucleotide substitutions or deletions/insertions of single nucleotides or of small oligonucleotides leading to frameshift. Rarely β-thalassemias are due to large gene deletions.[2]

β-thalassemias are prevalent in Mediterranean countries, the Middle and Far East, India, Southern China, and North Africa. The highest carrier frequency is reported in Cyprus (14%), Sardinia (10.3%), and Southeast Asia.[1] The high gene frequency of β-thalassemia in these regions is most likely related to the selective pressure from Plasmodium falciparum malaria.[1] Population migration and intermarriage between different ethnic groups has introduced thalassemia in almost every country of the world, including Northern Europe, where thalassemia was previously absent. It has been estimated that about 1.5% of the global population (80 to 90 million people) are carriers of β-thalassemia, with about 60,000 symptomatic individuals born annually, the great majority in the developing world. The clinical manifestations of β-thalassemias are heterogeneous ranging in severity, in the homozygotes or compound heterozygotes from the very severe transfusion-dependent anemia (thalassemia major) to the mild non-transfusion dependent thalassemia intermedia. The heterozygotes (thalassemia minor) are asymptomatic but identifiable by the reduced mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) and characteristically increased hemoglobin (Hb)A_2.[2] Clinical presentation of thalassemia major occurs between 6 and 24 months. Affected infants become progressively pale with feeding problems, diarrhea, irritability, recurrent of fever, and progressive enlargement of the abdomen caused by spleen and liver enlargement may occur. Without treatment, affected children have severely compromised growth and development and shortened life expectancy. Conventional management is based on regular red blood cell transfusions and iron chelation therapy to remove iron introduced in excess with transfusions. If a regular transfusion program that maintains a minimum Hb concentration of 9.5 to 10.5 g/dL is initiated, growth and development tends to be normal up to 10 to 12 years.[3] However, transfused patients, if not adequately chelated, may develop complications related to iron overload. Complications of iron

overload in children include growth retardation and failure or delay of sexual maturation. Later iron overload related complications include involvement of the heart (dilated myocardiopathy or rarely arrythmias), liver (fibrosis and cirrhosis), and endocrine glands (diabetes mellitus, hypogonadism and insufficiency of the parathyroid, thyroid, pituitary, and, less commonly, adrenal glands).[4] Compliance with iron chelation therapy mainly influences frequency and severity of the iron overload-related complications. Individuals who have not been regularly transfused usually die before the second to third decade. Survival of individuals who have been regularly transfused and treated with appropriate chelation extends beyond age of 40 years.

Individuals with thalassemia intermedia present later than thalassemia major, have milder anemia and by definition, do not require or only occasionally require transfusions. At the severe end of the clinical spectrum, patients present between the ages of 2 and 6 years, and although they are capable of surviving without regular blood transfusion, growth and development are retarded. At the other end of the spectrum are patients completely asymptomatic until adult life with only mild anemia.

β-thalassemias are the prototype of how a remarkable variability of disease severity can be generated in a single gene disorders. The type of mutation at the β globin locus is the most reliable and predictive factor of the disease severity, but genetic variants at or outside the β globin cluster, identified in continuous increasing number, are responsible for the wide phenotypic variability of the disease. This article reviews the molecular basis of the phenotypic diversity of the β-thalassemias with an overview of the genetic modifiers acting on -thalassemia heterozygotes and on homozygotes and genetic compound for -thalassemia alleles.

Several genetic modifiers have been identified able to modulate the disease clinical expression so that a classical monogenic disorder can be considered a multigenic disease. Genetic modifiers can be divided in *primary*, which modulate the clinical severity of the disease (this encompasses the complications of disease), and *secondary*, which affect some disease complications (Table 1).

Primary modifiers

In β-thalassemia, the reduced amount or absence of β globin chains results in a relative excess of free α globin chains in the bone marrow erythroid precursors, leading to their premature death and hence to ineffective erythropoiesis, which is the main determinant of the severity of the anemia. A minor contribute is due to peripheral hemolysis and overall reduction in hemoglobin synthesis.

The major variation of β-thalassemia is seen in the nature of the different mutations, which range from silent to dominantly inherited mutations.[5] The varying degree of globin chain imbalance resulting from the variable impairment of the β globin synthesis correlates with the disease severity. Since the pathophysiology of the disease is mainly related to the excess of impaired α globin chains, variations in α globin or γ globin production can greatly influence the disease manifestations (Figure 1).

Alpha globin variations

β-thalassemia and α-thalassemia occur at high frequency in many populations; therefore, it is not uncommon to find subjects who co-inherit both conditions.

Co-inheritance of α-thalassemia by reducing the α/non α globin chain imbalance tends to reduce the severity of the anemia in homozygotes or compound heterozygotes for β-thalassemia. The degree of amelioration depends on the severity of the β-thalassemia mutation and on the number of residual functional α globin genes. The effect is most apparent when only two α genes are functional and the β globin mutations are mild.[5-7] Rare patients have been reported who have co-inherited only one functional α globin gene (- -/- α Hb H genotype) with homozygous β-thalassemia: patients with homozygous β^+ thalassemia have thalassemia intermedia, while patients with homozygous β° thalassemia have a severe phenotype.[8,9]

β-thalassemia heterozygotes, whether β° or β^+, are completely asymptomatic and characterized by reduced MCV, MCH, increased Hb A_2, and mild imbalanced α/β globin chain synthesis. Co-inheritance of α-thalassemia tends to normalize MCV and MCH up to normal values and reduce the α/β synthesis, but Hb A_2 remains diagnostic in the β-thalassemia carrier range.[10,11] The clinically asymptomatic state of the β-thalassemia heterozygotes may be converted to that of thalassemia intermedia by the co-inheritance of triplicated or quadruplicated α globin gene arrangement, which by increasing the α globin production worsens the α/β globin chain imbalance.[10,12] However, the phenotypes resulting from this interaction are variable and related to the severity of the β-thalassemia allele.[13,14]

More marked hypochromia, microcytosis, and anemia has been reported in subjects with interacting heterozygous β°-thalassemia and single functional α globin gene (*i.e.*, Hb H genotype).[15]

Table 1. Genetic modifiers of β-thalassemia.

Primary modifiers:	
- α globin gene variations	
· α-thalassemia	
· excess α genes	
- γ globin variations	
· XmnI G-γ polymorphism	
· BCL11 SNPs	
· HBS1L-MYB SNPs	
Secondary modifiers:	
Bilirubin metabolism	
· UGT1A1 $(TA)_7$ motif	
Iron metabolism	
· HFE variants	
· GSTM1 null type	
Cardiac disease	
· (APOE)ε4 alleles	
· Some HLA haplotypes	
Osteoporosis	
· Vit D receptor polymorphisms	
· COLIA1, A2 polymorphisms	
· TGFB1	

Gamma globin variations

γ globin chains binding to α can substitute for β globin, reducing the excess of α globin and the premature red blood cell precursors death in the bone marrow.

Genetic determinants able to sustain a persistent production of γ chains in the post-natal life may ameliorate the clinical severity of homozygous β-thalassemia. The increase in γ chain output may depend on the type of thalassemia mutation *per se*, as it occurs in deletion or non-deletion δ-β-thalassemia. Co-transmission of heterozygous β-thalassemia with deletional hereditary persistence of fetal hemoglobin (HPFH) characterized by high hemoglobin F levels, results in a mild thalassemia intermedia phenotype. However, both conditions are rare and do not explain the variation in Hb F levels observed in the general population. For many years, relatively common genetic determinants of increased HbF production have been recognized linked and unlinked to the β-globin gene cluster.[16] Early studies found that a single nucleotide polymorphism (SNP), recognized by the XmnI restriction enzyme in the proximal promoter of HBG2 (G-γ globin gene -158 C→T), was associated with increased Hb F levels usually under conditions of stress erythropoiesis, such as sickle cell anemia and β-thalassemia.[17-19] Some β-thalassemia alleles, *i.e.*, codon 6 –A and codon 8 –AA, which are in linkage disequilibrium with the G -158 C→T polymorphism, are consistently associated with higher HbF production and a mild thalassemia phenotype. From genome wide association (GWA) studies, the trait variance attributed to this locus was 10.2% in Northern Europeans.[20,21] Linkage studies, candidate-gene association studies, and GWA studies led to map two quantitative trait loci (QTL_s) (HBS1L-MYB intergenic region on chromosome 6q23 and BCL11A on chromosome 2p16), modulating HbF expression.[20-22]

Different polymorphisms at the BCL11A gene on 2p16.1[22] and HBS1L-MYB intergenic region on 6q23.3 have been described.[5,23] Several polymorphisms within intron 2 of the BCL11A gene have been strongly associated with Hb F levels: rs766432, rs4671393, rs1427407, and rs11886868, all in high linkage disequilibrium (LD) with each other.[21,24-27] Other independent signals in the same area were also identified with rs10189857 and rs7599488, in high LD, as well as rs7606173 and rs6706648, also in high LD with each other.[25,26] In the HBS1L-MYB intergenic region, different SNPs have been described as being associated with Hb F variations in different studies: rs9399137, as well as rs4895441, rs9402686, and rs28384513.[23,25]

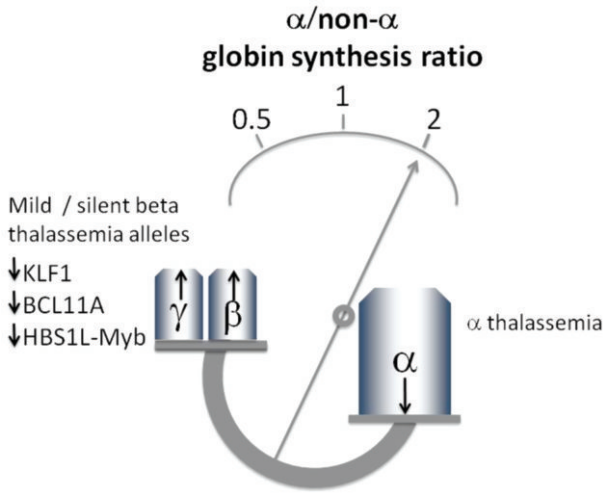

Figure 1. Primary gene modifiers in β-thalassemia. Modifying factors generally act by improving or worsening the α/non α biosynthetic ratio.

Together with Xmn 1 HBG2 polymorphism, the two previously mentioned loci account for 20-50% of the common variation in HbF levels in healthy adults, patients with sickle cell anemia, and β-thalassemia from different ethnic origins.[21,28,29] We reported the impact of variants in the BCL11A and HBS1L-MYB loci together with α gene defects on the clinical severity of β°39 thalassemia, quantifying their overall contribution to 75% of the variation differences between β°39 thalassemia major and intermedia phenotypes.[27] Badens *et al.*[28] extended previous studies by integrating the -158 C→T G-γ polymorphism and $β^°/β^+$ status, in addition to rs11886868 in the BCL11A gene, rs9389268 in the HBS1L-MYB intergenic region and α globin genes defects, to define β-thalassemia severity. Multivariate analysis, including these five genetic modifiers, was carried out and an accurate prediction has been made regarding major/intermedia status in more than 80% of patients. The respective contribution of known modifiers (α globin gene defects, XmnI HBG2 polymorphism, different SNP_s at the BCL11A locus and in the HBS1L-MYB intergenic region) to the prediction of β-thalassemia severity as assessed by the age at first transfusion, which accurately reflects variations in β-thalassemia phenotype severity, has been evaluated in a cohort of 316 homozygous β° thalassemia patients.[29] Results showed that the delay or absence in transfusion needs is mostly due to genetic variants affecting fetal hemoglobin production with an additional role of α-thalassemia.

It is important to outline that two QTLS include oncogenes (HBS1L-MYB and BCL11A), emphasizing the relevance of cell proliferation and differentiation as an important contribution to the HbF modulation. Biological studies of these QTLs provide data supporting long-held views on the mechanisms of hemoglobin switching (*i.e.*, changes in trans-acting factors and perturbation of the erythropoiesis kinetics).[30,31] Specifically, *in vitro* and animal studies indicate that BCL11A is able to silence γ globin genes, thereby actively participating in the switching process in co-operation with other transcriptional factors.[32] One important partner interacting with BCL11A is KLF1, a zinc finger erythroid transcription factor that in addition to activate the β globin gene, binding to the critical β gene CACCC box promoter element, directly downregulates the BCL11A gene.[33,35] Several KLF1 mutations have been reported associated with increased HbF in adult life and delayed hemoglobin switching.[35-38]

Recent studies have shown that polymorphisms in the olfactory receptor gene region mapping upstream of the β-globin gene cluster LCR are associated with HbF concentration in sickle cell anemia.[39] This region may modulate HbF levels by an effect on the chromatin structure of the β-globin gene cluster. On the other hand, suggested

loci on Xp22.2-22.3 and 8q26-28 outside the β-globin gene cluster, previously found to affect HbF, were not supported by studies.[20,21,40,41] The challenge now is to delineate all the causal variants and the exact mechanisms underlying the control of HbF expression, hoping to identify new potential targets (*i.e.*, induction of fetal hemoglobin) for a desirable therapeutic approach for patients with major hemoglobinopathies.

Extended molecular diagnosis can be carried out in patients or fetuses affected by homozygous -thalassemia to understand their phenotypic modulation abilities better. This information may have implications for genetic counseling and for therapeutic decisions (*i.e.*, when and if starting transfusion therapy).

Secondary modifiers

The clinical phenotype of homozygous β-thalassemia may also be modified by the coinheritance of other genetic factors mapping outside the β-globin gene cluster and affecting some disease complications. Among these factors, the ones best delineated so far are those affecting bilirubin, iron, and bone metabolisms.[42] Because of the rapid turnover of red cell precursors and the resulting breakdown of the heme products, both homozygotes and heterozygotes for β-thalassemia may develop mild jaundice and have the propensity to gallstone formation. In transfused thalassemia major patients, a further increase in bilirubin production results from the breakdown of transfused red blood cells. It has been shown that in these subjects, the level of indirect bilirubin, as well as gallstone formation, are related to a polymorphic motif in the promoter in the gene involved in the hepatic glucuronidation of bilirubin, namely bilirubin UDP-glucuronosyltransferase (UGTlA1). In normal individuals, the UGTlA1 promoter has six TA $(TA)_6$ repeat in the TATA box. Homozygotes for an additional repeat $(TA)_7$ develop mild unconjugated hyperbilirubinemia (Gilbert's syndrome) because of less efficient activity of UGTlA1. Studies in thalassemia major, thalassemia intermedia, and the β-thalassemia carrier state have shown that hyperbilirubinemia, and gallstones are correlated /are associated with the less efficient $(TA)_7$ motif.[43-48] It is clear from these findings that the Gilbert's syndrome associated mutation acts as a modifying gene in β-thalassemia by determining or promoting the development of jaundice or gallstones.

Patients with β-thalassemia, especially when not adequately chelated, develop iron overload in many organs, including liver, heart, and endocrine glands, in part because of the destruction of transfused red blood cells and in part, particularly in thalassemia intermedia, because of increased iron absorption. Some studies seem to indicate that the common mutation of the HFE gene (C282Y), which causes the common type of hereditary hemochromatosis (HH), might be involved in determining the variability of iron overload in patients with thalassemia intermedia.[49] However, in thalassemia major, the presence of a single mutation in HFE gene (C282Y and H63D) does not influence the severity of iron loading assessed by serum ferritin and liver iron concentration, likely because the effect of the mutations on iron overload is hidden by the treatment (*i.e.*, post-transfusional iron overload and iron chelation).[50] Furthermore, homozygosity for the H63D mutation whose functional significance in HH is still being evaluated, when coinherited with heterozygous β-thalassemia, seems to determine an increase in iron overload.[51] Conversely, coinherited heterozygosity for β-thalassemia appears to increase the rate of iron accumulation in C282Y homozygotes.[52]

Cardiac disease is the leading cause of death in patients with thalassemia major as a result of heart failure or arrhythmias. Pathophysiology of heart disease is multifactorial reflecting iron overload, chronic anemia, and pulmonary hypertension but genetic factors play a role. Apolipoprotein E ε4 allele and some HLA haplotypes, seem to be genetic risk factors for left ventricular failure in homozygous β-thalassemia.[53,54] Recently, a polymorphism in glutathione-S transferase M1 gene has been associated with an increased risk of heart iron overload in thalassemia major.[55] Another common complication in adults with β-thalassemia is the development of marked and progressive osteoporosis, which depends on many factors, including hypogonadism and the extent of iron chelation. However, recent evidence seems to indicate that the development of this complication may be also related to polymorphisms of the genetic loci involved in bone metabolism, namely vitamin D receptor, COLIAI gene, COLIA2 gene, and TGFB1, even though these results have not been confirmed in other studies.[56-61]

Conclusions

β-thalassemias are common disorders with a wide clinical spectrum and genetic heterogeneity. Recent progress in DNA analysis has made available a huge amount of information on genetic determinants able to influence the phenotype of patients and carriers. Several primary and secondary genetic modifiers have been identified, but in a number of cases and for some disease-related complications, the responsible molecular mechanism has not been so far elucidated. Even though phenotype prediction from genotype is not always accurate, the information obtained from extended genetic analysis may be used for planning appropriate management and providing adequate genetic counseling, and may reveal potential new targets for therapeutic intervention.

An integrated approach by clinicians, geneticists, clinical researchers, and basic scientist is needed to improve further the knowledge of the variability and the treatment of patients with hemoglobinopathies.

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