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Thalassemia — From Genotype to Phenotype

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Abstract

Thalassemia encompasses serious diseases with complex pathophysiology that is difficult to explain since it is considered a group of defects with similar clinical effects, still not a single disorder.

Understanding genetic factors contributing to the pathophysiology of thalassemias has enabled the identification of causative genes and development of diagnostic tests, helping defuse the confusion that evolves from clinical descriptions alone and correlating clinical symptoms with thalassemia disease.

More than 300 disease-causing mutations have been identified so far, mostly behaving as Mendelian recessives, however, there are variants that cause a disease phenotype even when present in a single copy. The remarkable technical developments of molecular biology gradually make it possible to define many of the globin gene molecular underlying pathologies.

Still, current morbidity and mortality remain unacceptable underlining the need for further research in this area.

In this chapter, we have summarized the current state of knowledge in the field of molecular lesions that underlie thalassemias, how they relate to their phenotypes, as well as the importance of conveying to the reader the extent to which it is possible to explain their clinical heterogeneity at the molecular level.

Keywords: Thalassemias, genotype/phenotype correlation, molecular heterogeneity

1. Introduction

During the past few years there has been a rapid increase of knowledge in the field of genetic control of hemoglobin synthesis in health and disease, which led to reviving the interest in thalassemia and associated disorders of hemoglobin (Hb) production.

Interest in the Hb molecule arose from its changes in a series of common clinical intermingled conditions, caused either by its structural variants (hemoglobinopathies), defects of synthesis (thalassemias), or a diverse group of defects in the developmental progression from fetal to adult hemoglobin production (HPFH) [1].

The revelation of the molecular basis of thalassemia led to the concept of “molecular pathology and molecular medicine”, with a shift of *emphasis* from illness in patients or their organs to pathological affection at the cellular and molecular level. Furthermore the study of these disorders shows great promise of answering some fundamental questions about the genetic mechanisms involved in defective protein synthesis [2].

Thalassemias are a group of inherited microcytic, hemolytic anemias characterized by defective Hb synthesis. It is now clear that thalassemias occur much more frequently, and in more racial groups, than was previously realized. Thalassemias confer a degree of protection against malaria, due to the blood cells' easy degradation. This selective survival advantage of carriers or heterozygous advantage may be responsible for the perpetuation of the mutation in some populations [3].

Generally, thalassemias affect all races, particularly people of Mediterranean origin, Arabs, and Asians with variable incidences, where the highest reported rate is in the Maldives (18% carriers' rate), followed by that of descendants from Latin American and Mediterranean countries while the reported incidence rate is very low in Northern Europe (0.1%) and Africa (0.9%) [3,4].

Acquired abnormalities of hemoglobin synthesis may also arise as a secondary manifestation of hematologic neoplasia and can be seen in any population. However, it is more readily recognized where inherited hemoglobin abnormalities are rare and less likely to cause diagnostic confusion [5].

This high prevalence of thalassemia makes it one of the major health problems and a priority genetic disease. Treatment of β -thalassemia, albeit more and more available, still represents a significant drain of the country's resources due to the disease's major complications. A prevention program would be useful to overcome these problems, but it requires a preliminary knowledge of hemoglobin, disease pathophysiology, as well as a spectrum of globin gene mutations among different populations [6].

2. Pathophysiology

Thalassemias are caused either by variant or missing genes affecting hemoglobin (Hb) production.

Human hemoglobin is formed of four peptide (globin) chains, and these chains are differentially produced during ontogeny; hence, they are diverse at the embryonic, fetal, and adult stages [1].

Reviewing the genesis of the normal post-embryonal hemoglobins can facilitate understanding thalassemias.

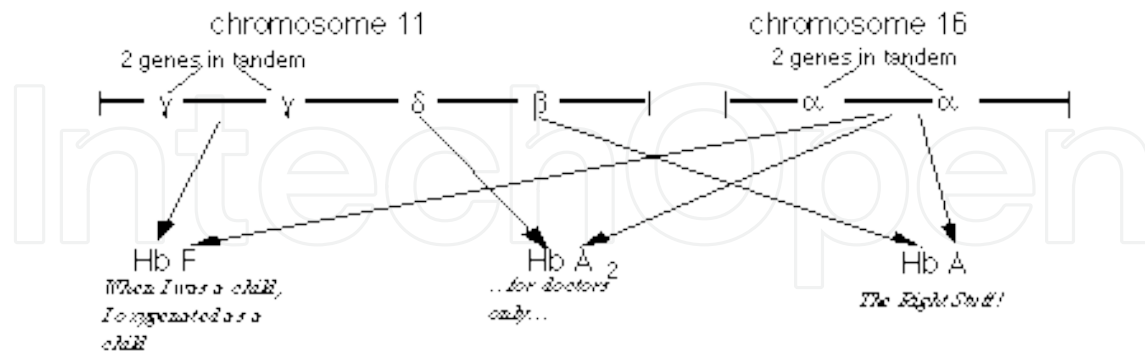


Figure 1. Normal adult human hemoglobins [7]

During embryonic development, zeta and epsilon (ζ & ϵ) globin chains disappear at 8 weeks to be replaced by α chains. In fetal life, Hb F ($\alpha_2\gamma_2$) is the predominant Hb. The whole Hb transition is completed by 6 months of postnatal life, with 97% Hb A ($\alpha_2\beta_2$), 3% or less Hb A₂ ($\alpha_2\delta_2$), and up to 1% Hb F (Fig. 1) in a normal adult [8]. These transitions among different hemoglobins are a reflection of the physiological adaptations to differing oxygen requirements at various stages of development.

The normal adult's Hb molecule is a spheroidal protein that contains two pairs of different globin molecules. It consists of four protein subunits arranged like a thick-walled shell with a central cavity forming an ellipsoidal tetrahedron (Fig. 2).

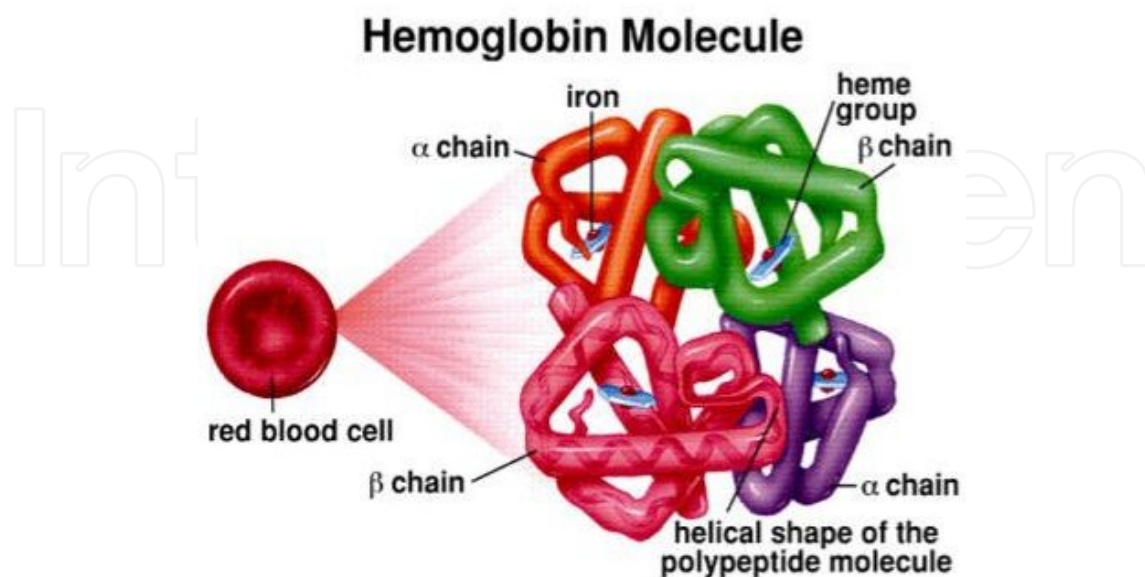


Figure 2. The structure of hemoglobin (Hb) [10]

The four subunits are two pairs of identical polypeptide chains: a pair of identical α or α -like chains and a pair of identical non- α or non- α -like chains. Each chain is associated with one haem molecule on the interior of the shell that is essential for oxygen uptake and release. The two α -like chains contain 141 amino acids while the non- α -like chains contain 146 amino acids [9].

The structure of globin genes has been highly conserved throughout evolution. Their transcribed regions are contained in three exons, separated by two introns. From the CAP site, the start of transcription, the first exon encompasses ~50 bp of 5' untranslated sequence (UTR) in which a TATA, CAAT, and duplicated CACCC boxes and a major regulatory region, containing a strong enhancer, maps 50 Kb from the beta globin gene.

Exon 1 encodes also codons for amino acids 1–31 in the α - and 1–29 in addition to the first two bases of codon 30 in the β -globin genes [9]. While exon 2 encodes amino acids 32–99 in the α - and 31–104 in the β -globin genes, that is the portion of the globin polypeptide involved in haem binding and the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) contacts. The remaining amino acids (100–141 for α , 105–146 for β), together with a 3' untranslated region of ~100 bp, are encoded in exon three [9] (Fig. 3).

In α -globin genes both introns are small, 117–149bp. The first intervening sequence in β -like genes is also small, 122–130bp, while IVS2 is much larger, 850–904bp. The removal of the intervening sequences from the initial transcript and joining the exon sequence to form mRNA is dependent on consensus sequences of exon-intron boundaries. At the 5' end of each intron the consensus is CAAG/GTAGAGT, where the excision site immediately precedes the invariant GT residues, the splice donor site. At the 3' end, the acceptor site, an invariant AG dinucleotide within a looser consensus (CT)N CT AG/G precedes the excision site and terminates the codon [9]. These consensus sequences are strongly upheld and in all cases GT and AG dinucleotides are maintained. As mutations in these sequences frequently lead to thalassemia, the globin genes provide excellent examples of their functional importance [9].

The expression and regulation of globin genes depend on the interactions of different trans-acting regulatory promoters, a series of enhancing elements and a “master” regulatory region involved in regulating the entire α - or β -globin gene complex. Widely expressed “housekeeping” genes that lack tissue specificity and maintain an early replicating, open chromatin structure in both erythroid and non-erythroid cells surround the α cluster. There are several upstream DNaseI hypersensitive sites (HSs), four of which are erythroid specific. Only one of these sites, 40kb upstream from the ζ globin gene, has strong enhancer activity (HS-40); this site is believed to be the major α globin gene regulatory element. It lies in an intron of a neighboring widely expressed gene (C16orf35) of unknown function. Knowledge of interactions of these sequences with both erythroid-specific and more generally active regulatory proteins is continuously acquired, however, details about how these complex interactions underlie the control of the globin-gene clusters remain to be worked out [9].

Ultimately, a very tightly controlled globin chain production process keeps the ratio of α - chains to non- α - chains, at 1.00 (± 0.05). Thalassemia, by altering this process, disrupts this ratio [11].

The basic pathology in all forms of thalassemia is a result of the presence of excess unstable globin chains within the affected RBCs. The effects of unstable hemoglobin chains on the

RBC membrane components are not the same and the pattern and rate of their precipitation differs [12].

Unlike the surfeit of α -chains that is produced in β -thalassemia, excess γ and β chains that result from defective α -chain production are able to form soluble homo tetramers, γ_4 (or Hb Bart's) and β_4 (or Hb H), which are physiologically useless because of their very high oxygen affinity as well as their instability in case of Hb H [9]. The clinical features of the more severe forms of α -thalassemia are therefore considered a reflection of hemoglobin Bart's and H properties as well as their effects on erythropoiesis, particularly on red-cell survival [9,13].

The unbalanced Hb synthesis, which is the principle of the pathophysiology of thalassemia, is caused by decreased production of at least one of the major globin polypeptide chains: alpha (α), beta (β), gamma (γ), delta (δ), and *epsilon* (ϵ).

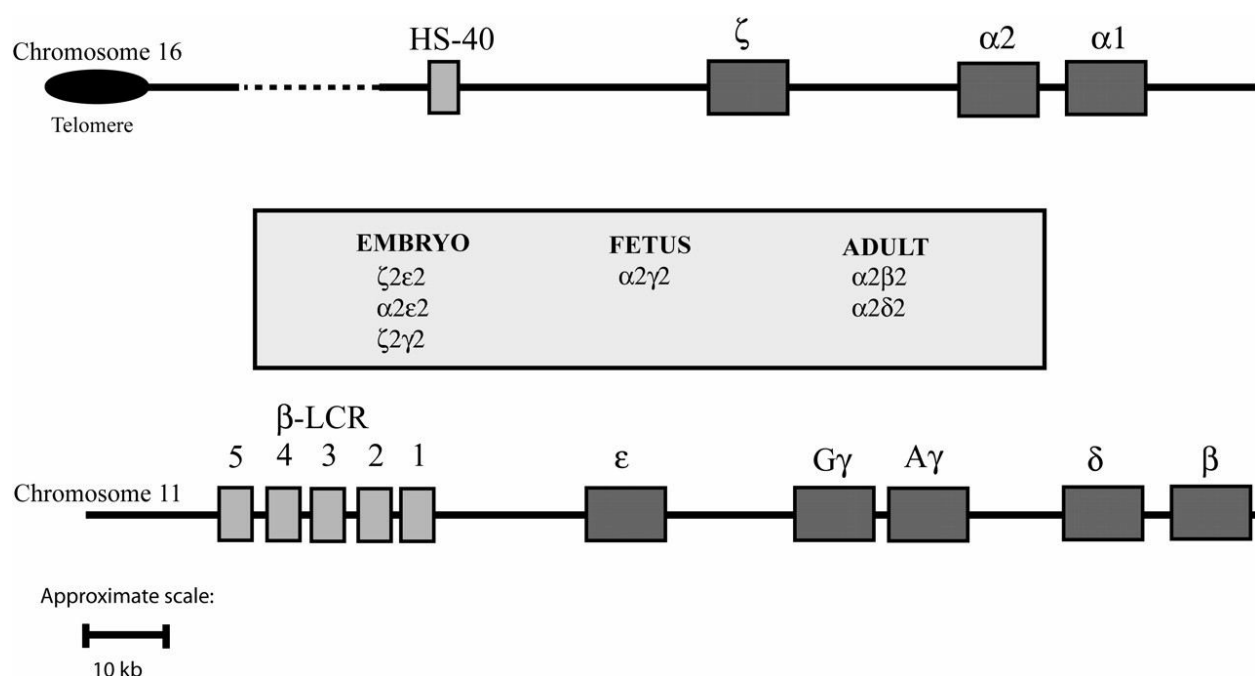


Figure 3. Schematic representation of the human globin gene clusters. ζ , $\alpha 2$, and $\alpha 1$ globin genes situated near the telomeric region of the short arm of chromosome 16. About 40 kb upstream of the 5' end of the ζ globin gene is the HS-40 α -globin genes remote regulatory region. The β -like globin cluster is located on the short arm of chromosome 11; upstream of the cluster is the β -globin gene remote regulatory locus control region (β -LCR). In between are represented the different ontogenetic stages of Hb [14]

3. Different types of thalassemia

- **α -Thalassemia** results from decreased alpha globin chain production. It is most commonly inherited as a recessive Mendelian disorder. Acquired α -thalassemia was reported under rare circumstances [14]. The production of α -globin chains is directed by two functional alpha genes ($\alpha 1$ and $\alpha 2$) located in the α -globin gene cluster at the short arm of chromosome 16p13.3 [15], which comprises two $\psi\alpha$ - genes followed by two α -globin genes [Fig. 4] in the same order as their expression throughout development [16].

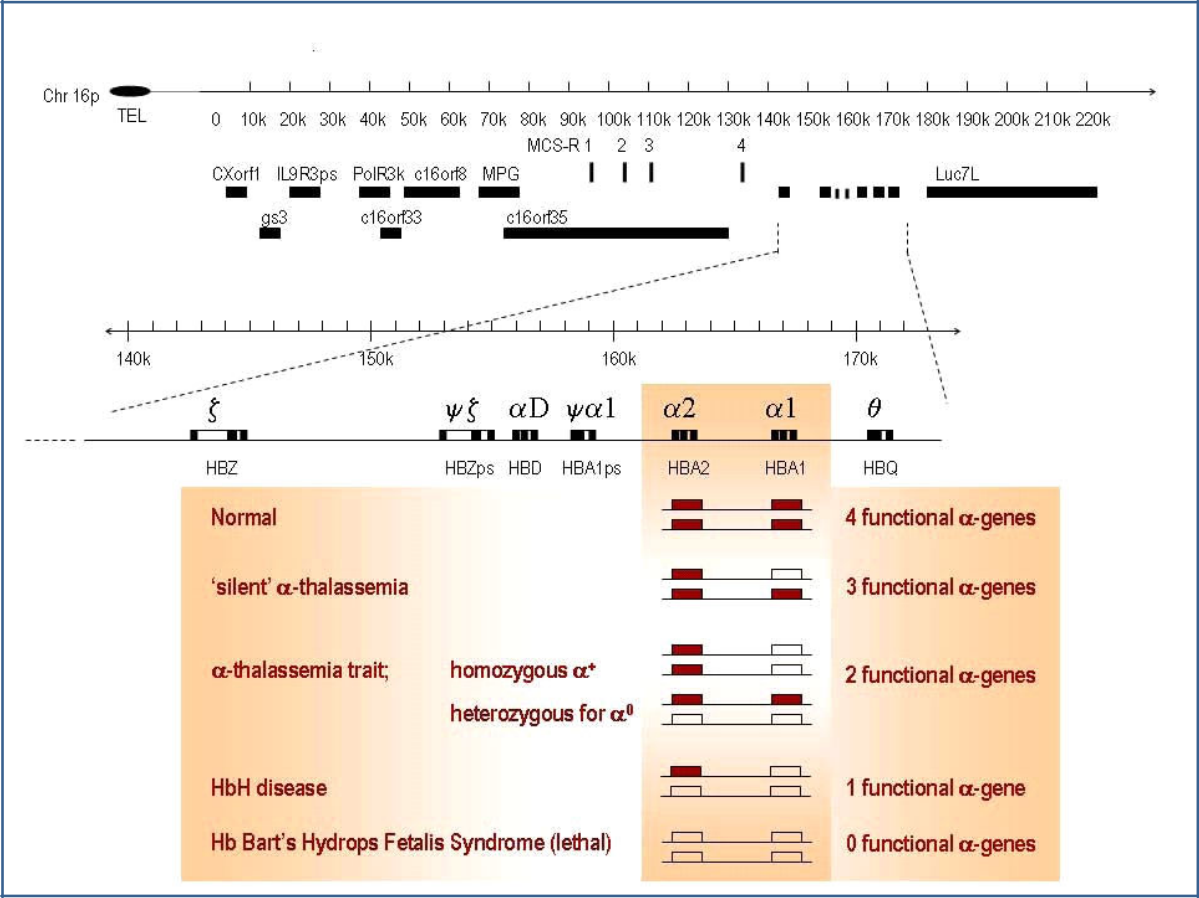


Figure 4. α -globin gene cluster on chromosome 16. The telomere is shown as an oval, genes are shown as boxes, and α -globin regulatory region (MCS-R 1 to 4) is indicated as vertical bars. The genotype/phenotype relationship is shown in the entailed table [17]

Decreased production of alpha2-globin or alpha1-globin gene products yields a relative excess of beta chains, which results in less stable chains with consequent clinical disease [18,19].

The severity of α thalassemias is correlated with the α globin genes affection; deletional or nondeletional mutations involving one (α^+) or both (α^0) alpha genes *in cis* at the α gene cluster resulting in four clinical phenotypes of α -thalassemia. The silent carrier state is caused by heterozygosity to the α^+ defect. However, α -thalassemia minor is caused by either homozygosity to the α^+ defects or heterozygosity to the α^0 defects [20].

Defects in 3 of the 4 genes severely impair α -chain production, resulting in tetramers of excess β chains (Hb H) or, in infancy, γ chains (Bart's Hb). Defects in all 4 genes are a lethal condition in utero as the lack of α chains affects O_2 transport [19].

- **β -Thalassemia** results from decreased production of β -polypeptide chains (β^+), or β^0 where no beta globin is produced. A less severe form β^{++} thalassemia denotes mild defect in β -chain production [21].

β -globin is encoded by a structural gene found in a cluster with other α -like genes spanning 70 Kb on the short arm of chromosome 11 (11p15.4) (Fig. 3). Beta thalassemia inheritance is

autosomal recessive: Heterozygotes are carriers and have asymptomatic mild to moderate microcytic anemia (thalassemia minor); homozygotes develop variable phenotypes, ranging from the severe transfusion dependent thalassemia major to the mild form of thalassemia intermedia [21]. The diagnostic feature of β -thalassemia is an elevated level of HbA2 in heterozygotes, found in most forms of β^0 and β^+ thalassemia. However, there are less common cases of normal HbA2 β -thalassemia with normal HbA2 level in heterozygotes [9].

- **δ -thalassemia** – about 3% of adult hemoglobin is made of α and δ chains. Mutations affecting δ -globin gene produce δ^0 or δ^+ thalassemia [22]. Mutations in the δ -globin gene are not pathologically relevant [23]. However, since high HbA2 levels are diagnostic for beta-thalassemia trait, co-inheritance of δ - and β - gene defects or **β - δ -thalassemia** may lead to misinterpretation of diagnostic results through preventing an elevation of the level of HbA2 [23].
- **HPFH** is characterized by persistent fetal hemoglobin synthesis in adult life in the absence of major hematological abnormalities. HPFH can modify the clinical phenotype of the β -thalassemia and reduce their severity. Many forms of HPFH are considered as extremely well compensated forms of β - or $\delta\beta$ -thalassemia [23,24]. In some forms there is no δ or β chain production and almost complete compensation by a high output of γ chains (**$\delta\beta$ 0 HPFH**). In other forms, the β - and δ -chain synthesis is directed by genes on the same chromosome, to the HPFH determinant, hence designated $^G\gamma\beta^+$ or $^A\gamma\beta^+$ HPFH, depending on the structure of the Hb F [9].

Another heterogeneous group of HPFH is present in otherwise normal individuals with much lower levels of Hb F. Evidence is mounting that the genetic determinants for some types of this form of HPFH are not linked to the β -globin gene cluster [9].

- **$\epsilon\gamma\delta\beta$ -Thalassemia** is a rare disease that results from loss of either the whole or a major part of the β -like globin-gene cluster and its regulatory regions. Strictly speaking, therefore, it should be described as ($\epsilon^G\gamma^A\gamma\delta\beta$) 0 thalassemia. Homozygotes would not be compatible with life; heterozygotes have the clinical phenotype of β -thalassemia with a normal HbA2 level [9].
- **γ -Thalassemia** is not associated with imbalanced globin production and hence does not appear to be of clinical significance. Deletions involving one or more of the γ -globin genes have only been identified during surveys of the relative levels of $^G\gamma$ and $^A\gamma$ chains in Hb F. Only one form of γ -thalassemia has been described due to about 5 Kb deletion at the 3' end of the $^G\gamma$ gene, the 5' end of the $^A\gamma$ gene and the intergenic region. Most likely it resulted from unequal cross overs between $^G\gamma$ and $^A\gamma$ resulting in - $^GA\gamma$ -hybrid gene [2,25].
- **Thalassemia due to inheritance of structural Hb variant**

Hemoglobin E/thalassemia: Common in India, Bangladesh, and throughout Southeast Asia and although previously rarely diagnosed in North America or Europe, it has become the most common form of β -thalassemia detected through many newborn screening programs. It is clinically characterized by marked variability, ranging from mild asymptomatic anemia to a life-threatening disorder requiring transfusions from infancy [2,26].

Hemoglobin S/thalassemia is common in African and Mediterranean populations. Coinheritance of α or β -globin gene mutations can modulate the hematological diagnostic data and

clinical expression of the sickle cell. The phenotype of the β -globin gene defect determines the severity of the co-inherited sickle cell mutation; β^0 results in a severe disease, while β^+ causes a milder clinical picture of the disease [27]. Its coexistence with α -thalassemia (α^{thal}) lowers MCV & MCH, resulting in milder anemia, but causes a reduction in hemolysis and increase in total hemoglobin, which makes patients more prone to vaso-occlusive and painful crises [28].

Hemoglobin C/thalassemia is common in Mediterranean and African populations. HB C usually results in mild asymptomatic anemia; however, hemoglobin C/ β^0 thalassemia can cause moderately severe hemolytic anemia with splenomegaly while hemoglobin C/ β^+ thalassemia produces a milder disease [29].

Hemoglobin D/thalassemia is common in the northwestern parts of India and Pakistan (Punjab region). Hb D homozygotes are asymptomatic. Coinheritance of Hb D/ β -thalassemia results in mild anemia \pm mild splenomegaly while coinheritance with α -thalassemia results in higher Hb levels [30].

4. Molecular pathology/relevance to phenotype

Thalassemia is a difficult disease to explain, since it is considered a group of defects with similar clinical effects, still not a single disorder.

Molecular characterization of thalassemia is of great importance in helping defuse the confusion that evolves from clinical descriptions alone. Therefore, the best approach is to understand the genetic background in correlation with clinical symptoms of patients with thalassemia disease.

The remarkable technical developments of molecular biology gradually make it possible to define many of the globin gene molecular underlying pathologies. Herein we will try to summarize the molecular lesions that underlie thalassemias, how they relate to their phenotypes, as well as the importance of conveying to the reader the extent to which it is possible to explain their clinical heterogeneity at the molecular level.

The molecular basis of the thalassemias has been scrupulously expounded and represents one of the first diseases to be characterized at the molecular level.

Different mutations; point mutations, small insertions, deletions, or, in some cases, partial or large deletions encompassing one or two globin genes alter the function of the genes encoding a globin chain [31].

For example, mutations resulting in the absence of β -chain synthesis (β^0 -thalassemia) are at times caused by β -globin gene deletions or more commonly by subtle mutations such as nonsense, frame shift, or RNA-splicing mutations [31]. While in β^+ thalassemia with only reduction in β chain synthesis, β -globin gene mutations may be located in promoter regions, at exon-intron boundaries disrupting splice site recognition, within introns generating cryptic splice signals or farther downstream at sites that regulate RNA stability [31].

On the other hand, non-deletional α -thalassemia mutations are not as prevalent as the deletional mutations. However, they were reported in some regions with high consanguinity rates such as in Saudi Arabia and the surrounding Gulf countries [31]. Deletion mutations affecting one, two, or three α -globin genes yield mild to severe hemolytic anemia. Deletion of all four α -globin genes results in homozygous α -thalassemia clinically manifesting as hydrops fetalis, a condition associated with stillbirth [31].

Genetic classification of α -thalassemia

Analysis of the human α -globin cluster has revealed a remarkable degree of structural variability due to point mutations, deletions, and insertions of DNA.

The α -globin genes are embedded within two highly homologous 4 kb duplication units, the sequence identity of which has been maintained throughout evolution by gene conversion and unequal crossover events [9]. These regions are divided into homologous sub-segments (X, Y, and Z). α -thalassemia is classified according to its cause into deletional and non-deletional as follows.

α^+ -thalassemia due to deletions ; α -thalassemia is more frequently caused by deletion. One of the most common α -thalassemia deletions is the rightward deletion due to reciprocal recombination between Z segments that are 3.7 kb apart, producing a chromosome with only one α -globin gene ($-\alpha^{3.7}$, rightward deletion) causing α -thalassemia and α -triplication allele without a thalassemic effect (Fig. 5).

Another common crossover between homologous X boxes, which are 4.2 kb apart, also gives rise to an α -thalassemia determinant ($-\alpha^{4.2}$) and an α -triplication allele anti4.2 chromosome [32]. Further recombination events could give rise to other unusual rearrangements. Other rare deletions that produce α^+ thalassemia have also been described, for example, deletion of the entire α -1 gene and its flanking DNA ($-\alpha^{3.5}$) [Fig. 6] [9, 33].

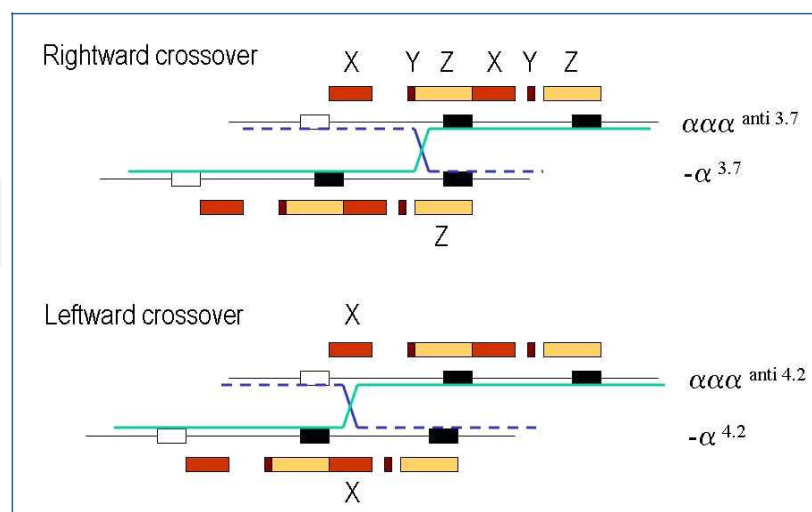


Figure 5. Schematic representation of α^+ -thalassemia. $\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\ 3.7}$ chromosomes (top) resulting from rightward cross-over between the mis-paired Z boxes during meiosis. $-\alpha^{4.2}$ and $\alpha\alpha\alpha^{anti\ 4.2}$ chromosomes (bottom) resulting from leftward cross-over between misaligned X boxes [33]

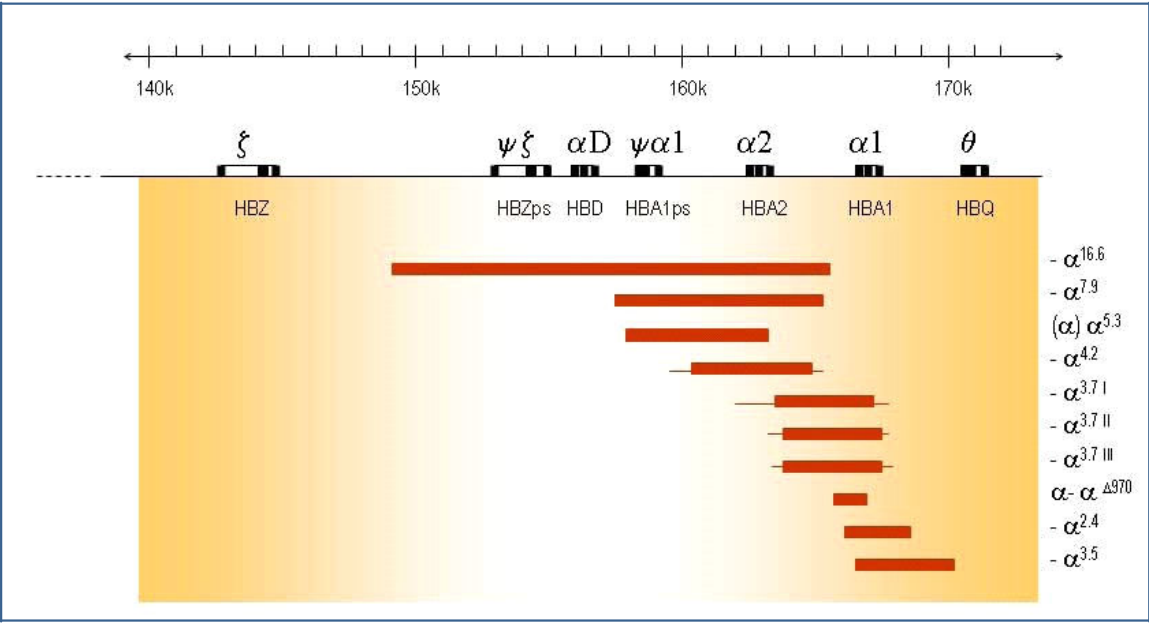


Figure 6. Deletions of one α -gene giving rise to α^+ -thalassemia. The extent of the deletion is shown as bars, and thin lines indicate regions of uncertainty of the breakpoints [33]

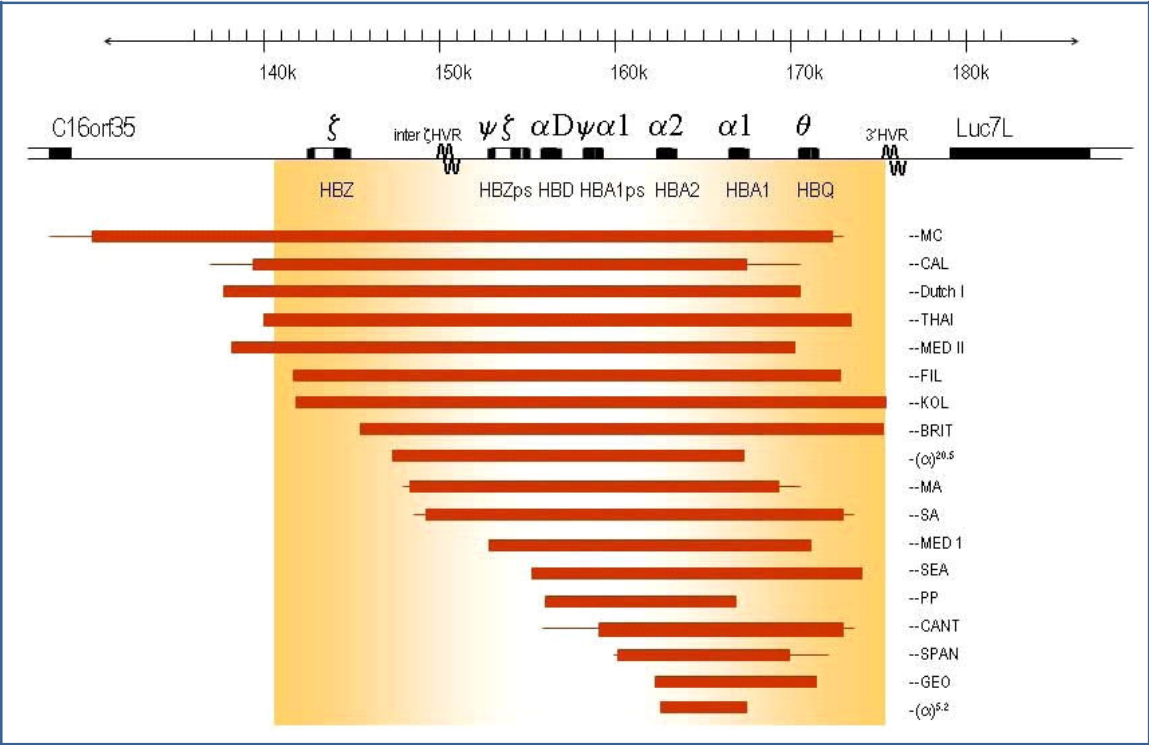


Figure 7. Deletions of two α -genes giving rise to α^0 -thalassaemia [33]

- **α^+ -thalassemia due to non-deletion types of α -thalassemia**, generally, the non-deletion α^+ - thalassaemia gives a more severe reduction in α -chain synthesis than the deletion type.

Many mutations have been described mostly affecting mRNA processing, translation, and α -globin stability. The most common non-deletional variants are the polyadenylation site mutations $\alpha^{2AATAAG}$, $\alpha^{2AATGAA}$, and α^{2AATA-} (Mediterranean and Middle East) [34,35].

- **α^0 -thalassaemia due to deletions** many different length deletions have been found in patients with α^0 thalassemia, which were named after whom or where they were first discovered or by deletion size. The complete or partial deletion of both α -genes in *cis* results in no α -chain synthesis directed by these chromosomes *in vivo*. Homozygotes for such deletions have the Hb Bart's (Fig. 7&8) hydrops fetalis syndrome, while compound heterozygosity to the α^0 and α^+ defects results in a phenotype of hemoglobin H disease [19].

Some rare deletions of the regulatory region cause α^0 -thalassaemia. This regulatory region lies 40–50 kb upstream of the α -globin gene cluster. This region composed of four multispecies conserved sequences (MCS), called MCS-R1 to R4, of which the most essential for α globin expression is MCS-R2 (HS-40) [36]. An overview showing all currently known $(\alpha\alpha)^T$ deletions is given in Figure 8 a and b.

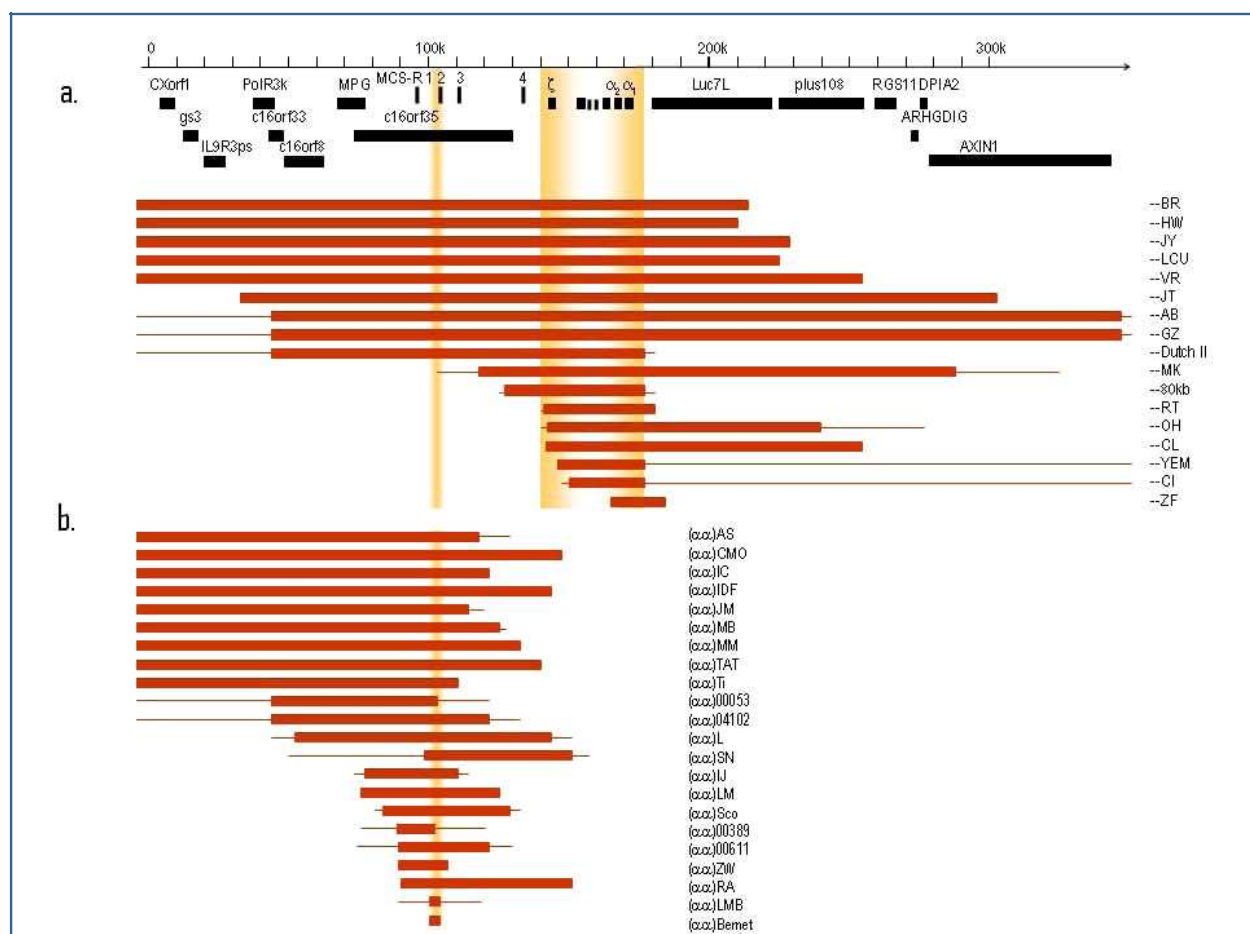


Figure 8. (a) Large deletions involving both α -genes and (b) deletions of the α -globin regulatory region leaving the α -genes intact [33]

α -thalassemia mental retardation X-linked (ATRX)

Hematologic malignancy and abnormal erythropoiesis may acquire changes in the hemoglobin structure or synthesis [Fig. 9]; however, the molecular basis of such abnormalities is obscure [5].

Somatic mutations in a known *trans*-acting regulator of globin gene expression were recently identified in patients with myelodysplasia, α -thalassemia mental retardation X-linked (ATRX). These new observations may increase the understanding of normal control of globin gene expression, as well as clarify common genetic or epigenetic mutations that contribute to the development or progression of myelodysplasia [5].

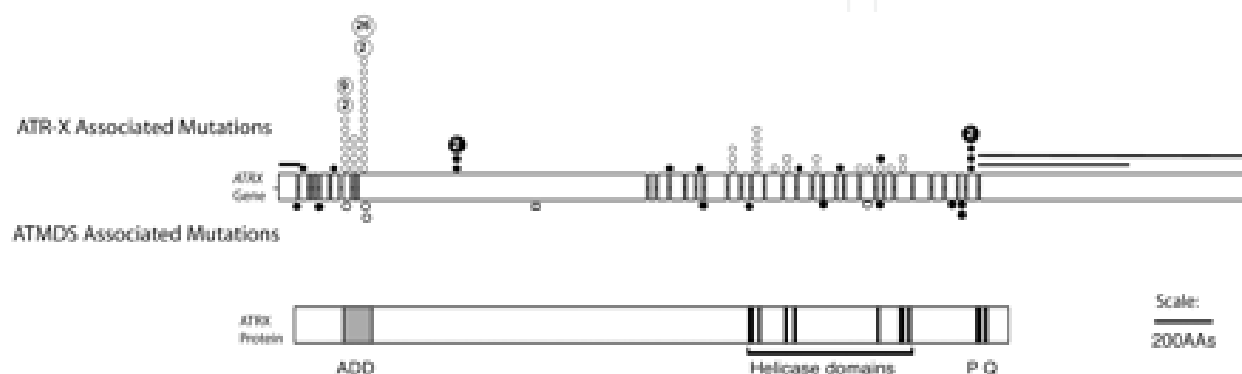


Figure 9. Schematic representation of the spectrum of ATRX mutations. Alignment of mutation sites in ATRX gene (top) with functionally important protein domains in ATRX protein (bottom) [5]

Genetic classification of β -thalassemia

The fact that only two genes encode the β -globin chain makes β -thalassemia simpler to understand than α -thalassemia. Unlike α -thalassemia, β -thalassemia rarely arises from the complete loss of a β -globin gene, but rather its suppression to variable degrees with essentially no beta globin protein production (β^0) or lower than normal (β^+). The severity of beta thalassemia depends in part on the type of β -thalassemic genes that a person has inherited.

- One β -globin gene affection; the residual production capacity of the defective beta globin gene tunes the degree of alpha-beta globin imbalance. One normally functioning beta gene, even if the other one produces no beta chain, would clinically present itself as mild anemia [37] that would be detected during a routine laboratory blood evaluation.

Two β -globin gene affection causes severe anemia and a potentially life-threatening condition. The severity of the disorder depends in part on the combination of genes that have been inherited: $\beta^0\text{thal}/\beta^0\text{thal}$; $\beta^0\text{thal}/\beta^+\text{thal}$; $\beta^+\text{thal}/\beta^+\text{thal}$, even though, the β^+ thalassemia genes vary greatly in their ability to produce normal hemoglobin.

β -thalassemias are heterogeneous at the molecular level. More than 300 disease-causing mutations have been identified so far. The distribution of β -thalassemia alleles varies highly from one population to another hence each population has its own spectrum of common β -thalassemia mutations that may be defined through a limited number of specific primers/

probes [38]. While most alleles behave as Mendelian recessives, there are variants that cause a disease phenotype even when present in a single copy.

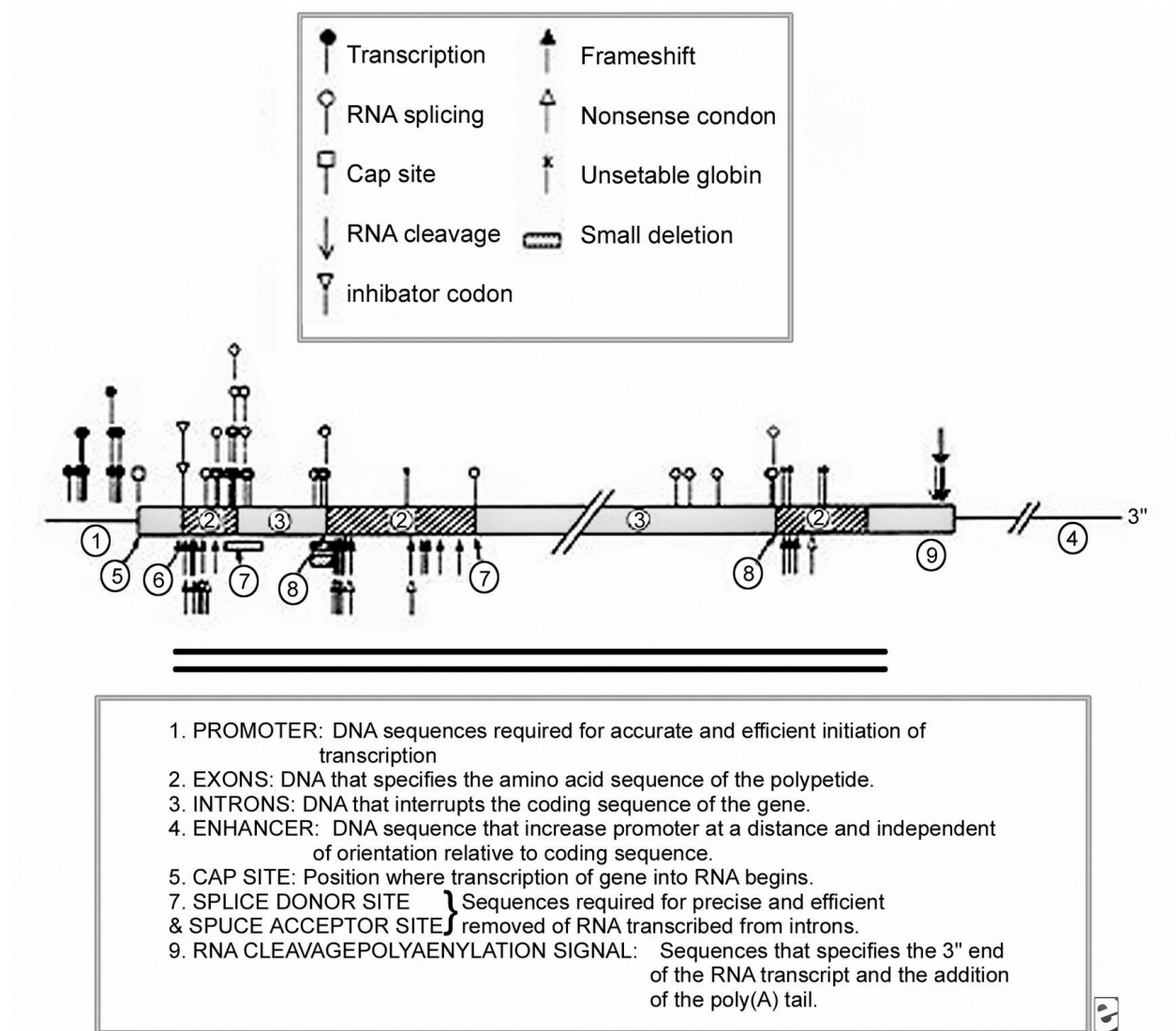


Figure 10. Beta globin gene mutations [39]

Most of reported beta globin gene mutations are single nucleotide substitutions or deletions or insertions (Fig.10) with consequent shift of reading frame. Gross gene deletion beta-thalassemias were reported in rare conditions. A complete updated list of beta-thalassemia mutations is available through the Globin Gene Server website [40]. β -thalassemia mutations may affect exonic intronic or the promoter (the 5' and 3' flanking UTR) sequences, accordingly affecting almost every known stage of the β -globin gene expression [23]. β -globin gene missense mutations, minor deletions leading to loss of intact codon, and frame shifts could all result in hyper unstable beta chains. Mutations producing the typical recessively inherited forms of β - thalassemia are located in exons 1 or 2 whereas most mutations in the phase termination codons that result in dominant beta-thalassemia lie in exon 3 [41].

In exons 1 or 2 mutations, a very small amount of beta globin mRNA is found in the cytoplasm of red blood cell precursors, whereas exon 3 mutations are associated with a substantial amount of abnormal cytoplasmic mRNA. This results in truncated unstable β - chain products, hence acting in a dominant-negative fashion, causing premature destruction of red blood cells. On the other hand, exons 1 and 2 premature termination codon mutations activate nonsense-mediated mRNA decay, thus precluding the accumulation of mRNA encoding for truncated peptides [41,42].

5. Different sorts of point mutations could affect β -globin expression

- **Transcriptional mutations:** described in each of the CACCC, CCAAT, and TATA boxes in the promoter region result in moderate reductions in transcription that give β^+ thalassemia with borderline reduced/normal red cell indices. Generally, such mutations are of β^+ or β^{++} mild forms of β -thalassemia.
- **RNA processing mutations:** the primary transcript of the β -globin gene is modified at the Cap site by the addition of a 7mG residue and at the 3' end by an extension of 20–50 adenine residues, poly-A tail. A mutation of the cap site (+1 A \rightarrow C) produces a mild form of β -thalassemia while numerous mutations of the poly-A addition signal result in more severe β^+ thalassemia in which normally modified mRNA is reduced to ~10% of normal [42].
- **Maturation of pre-mRNA:** if they fall into splicing or poly-adenylation sites. RNA splicing mutations represent a large portion of all β -thalassemia mutations; they affect the splicing process at variable degree, depending on mutation's position. Mutations occurring in the splicing consensus sequences are of β^+ type producing milder β -thalassemia. Whereas, mutations in exon or intron sequences may activate a cryptic splicing site leading to abnormal mRNA processing with defective splicing at variable degrees, and consequential mild to severe phenotypic affection [41,42].

Another group of splicing mutations involve nucleotide replacements within introns that create a new splice site that is used preferentially to the normal site. Depending on the strength of the new site these may result in either β^+ or β^0 thalassemia. Included in this group was the first characterized β -thalassemia allele, IVSI-110 G \rightarrow A, the most common allele in the eastern Mediterranean region [42].

New splice sites may also be created by nucleotide substitutions in exons. These may also produce an amino acid change that would then produce an abnormal globin chain, leading to the production of reduced protein amounts.

- **Mutations affecting RNA stability:** occur in the 3' or 5' UTR, Cap site or the poly-adenylation site and are generally associated with mild β -thalassemia phenotypes. 5' UTR mutations correlate with very mild phenotypes up to silent β -alleles with normal hematological phenotypes in heterozygotes [43].
- **Mutations affecting translation:** generate premature nonsense codons, premature termination of globin chain synthesis generally leads to the production of short, or to nonsense-

mediated decay (NMD) of abnormal mRNA. Six mutations of the translation initiation codon, AUG, have been described, all resulting in β^0 thalassemia. Downstream AUG sequences are not matched to the consensus sequence for translation initiation and would result in a shifted reading frame and a premature stop codon [44].

The deletion or insertion of one or a few nucleotides (other than multiples of three) into the coding region of the β globin gene causes a shift in the reading frame. These frame shift mutations will allow continued reading of an altered amino acid sequence until a new termination codon is reached, producing a truncated product that is non-functional [44].

6. Modifier genes and genotype-phenotype outcome

Phenotype-genotype studies aiming to unravel the clinical heterogeneity of beta thalassemia syndromes lead to the identification of a number of genetic factors that affect disease severity (Fig. 11 a&b).

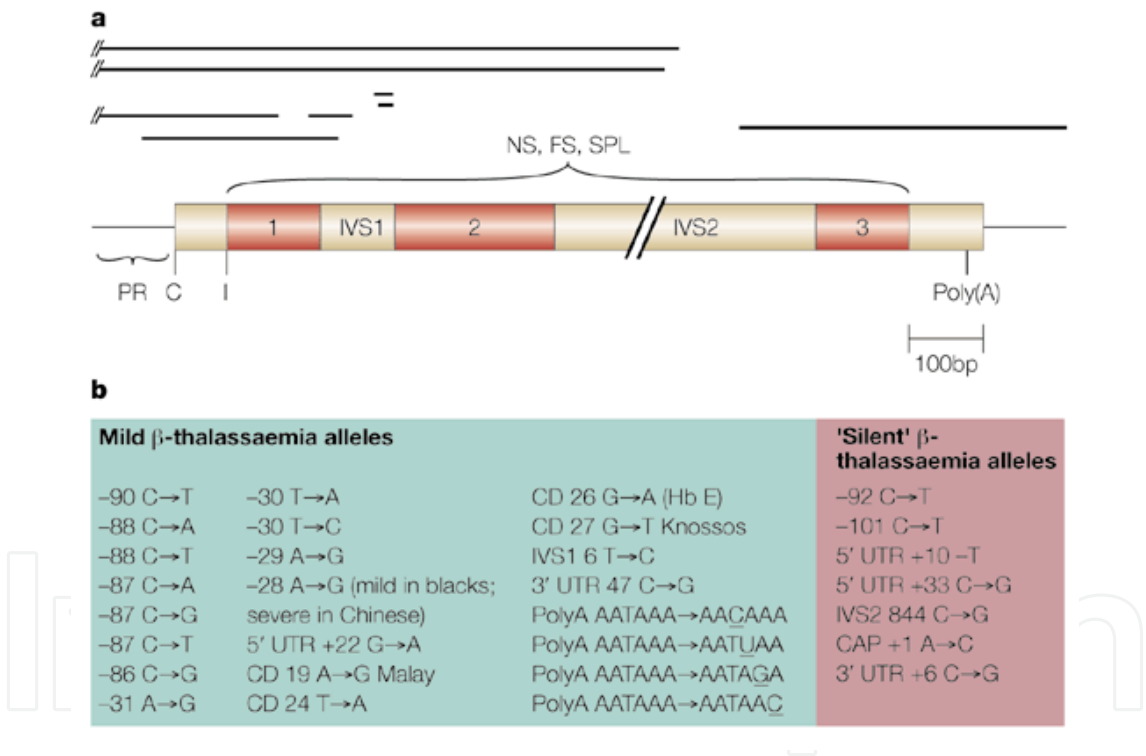


Figure 11. The effect of some beta globin mutations on phenotype.

Foremost among these is the nature of the β -globin mutations (primary modifiers) (Fig. 11). The major determinant of disease severity is the degree of β -globin chain deficit resulting from the nature of the β -thalassaemia alleles.

As the phenotype correlates with α -globin chain deficiency and improved α /non- α chain imbalance, an increased residual level of HbF in adult life compensates the decreased β -globin chain, hence a major determinant of disease severity [45,46].

In the last few years, by application of genome wide association studies (GWAS), three major single nucleotide polymorphisms (SNPs) known as quantitative trait loci (QTL) that affect Hb F production levels were identified [47].

The first QTL is a SNP of unknown functional significance at position -158 (C>T) of the γ promoter, called the XmnI polymorphism. The second locus is located on intron 2 of the BCL11A gene. The third locus is located between the HBS1L and MYB genes. It was suggested that the BCL11A and MYB proteins could act as γ -globin repressors [48].

Numerous polymorphisms located at the BCL11A gene on 2p16.18 and HBS1L-MYB intragenic region on 6q23.3 are either involved directly in fetal gene silencing in adult life or in cell proliferation and differentiation [49]. It was found that high-Hb F alleles at the BCL11A SNP (rs766432, rs4671393, rs1427407, and rs11886868) were significantly more frequent in patients with milder clinical forms of β -thalassemia and sickle cell disease (SCD), suggesting that this genetic polymorphism may be an important genetic modulator of disease severity [50]. Moreover, in the HBS1L-MYB intergenic region, different SNPs have been described as being associated with Hb F variations in different studies: rs9399137, rs4895441, rs9402686, and rs28384513 [51]. It was found that some particular tag-SNPs in these regions of favorable determinant are associated with high Hb F levels in healthy adults, as well as in thalassemia and SCD patients [52].

In $\delta\beta^0$ thalassemia, persistent production of gamma globin chains and their binding with α globin chains is an ameliorating factor. This ability is due to deletions of variable extents within the β -globin cluster, while in other cases it depends on the co-transmission of point mutations at $\Lambda\gamma$ or γ promoters (-196 C \rightarrow T $\Lambda\gamma$; -158 C \rightarrow T XmnI SNP) [53,54].

Genetic modifiers are progressively used to explain different phenotypic expressions in two patients harboring the same beta-thalassemia mutation. These modifiers comprise factors affecting α - and β -chains imbalance in β -thalassemia, mainly the association of α -thalassemia deletions or mutations, with associated alleles contributing to the persistence and/or increased Hb F production after birth [55]. The evidence of mild phenotypes may also be determined by coinheritance of genetic determinants associated with increased γ - chain production mapping outside the β -globin cluster. It is expected that the identification of the role of genetic modifiers that modulate Hb F levels will shed light on the molecular mechanisms that control Hb F expression and on the etiology of the clinical heterogeneity observed in SCD patients [51,55].

Three different studies concerned with the association between the latter two QTLs with β -thalassemia concluded that predictions based on genetic modifiers could foresee the major or intermedia type of β -thalassemia, even in cohorts of patients with various β -globin genotypes, and that identification of the functional Hb F-QTL SNPs should help improve the accuracy of β -thalassemia major/ β -thalassemia intermedia predictions [47,48,56].

Because of the major demographic changes in the pattern of disease in many of the developing countries, and the increased control of infant mortality due to diarrhea and infectious diseases, genetic diseases including thalassemias are floating on the surface as a health priority. Thus, translating the continuously growing molecular knowledge into readily understandable

molecular pathology and extending molecular medicine is of utmost importance for disease control and management [9].

Although nearly all globin mutations characterized to date have been *cis*-acting defects, it has recently emerged that globin synthesis may also be altered by *trans*-acting mutations involving erythroid-specific and general transcriptional regulators [14,57].

Variable mutations of the globin genes has empowered researchers to use this system to launch many of the fundamental principles of human molecular genetics trying to improve our understanding of molecular hematology [14]. Hemoglobin synthesis is an excellent model for understanding mammalian gene regulation.

Studying the entire globin clusters through whole genome and/or exome analysis, among continuously emerging technical approaches of molecular biology, may be possible in the near future. New technologies may also allow rapid and far-reaching outlooks of partner genes playing a role in the regulation of hemoglobin synthesis.

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