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Review

Molecular basis of α-thalassemia

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A B S T R A C T

α-Thalassemia is an inherited, autosomal recessive, disorder characterized by a microcytic hypochromic anemia. It is one of the most common monogenic gene disorders in the world population. The clinical severity varies from almost asymptomatic, to mild microcytic hypochromic, and to a lethal hemolytic condition, called Hb Bart's Hydrops Foetalis Syndrome. The molecular basis are usually deletions and less frequently, point mutations affecting the expression of one or more of the duplicated α-genes. The clinical variation and increase in disease severity is directly related to the decreased expression of one, two, three or four copies of the α-globin genes. Deletions and point mutations in the α-globin genes and their regulatory elements have been studied extensively in carriers and patients and these studies have given insight into the α-globin genes are regulated. By looking at naturally occurring deletions and point mutations, our knowledge of globin-gene regulation and expression will continue to increase and will lead to new targets of therapy.

1. Introduction

Human hemoglobin is a tetrameric protein consisting of two alpha-like and two beta-like globin chains each forming a pocket containing the heme group for binding oxygen (David Gell, this issue). The globin genes are arranged in two separate gene-clusters on different chromosomal loci in order of their expression during development (Fig. 1). Expression is regulated by complex interactions of transcription factors and regulatory elements (promoters and enhancers) to switch on and off genes in a stage specific and tissue specific manner.

Globin gene disorders (hemoglobinopathies) are characterized by either abnormal globin chain variants like sickle cell anemia or reduced globin chain synthesis in erythroid cells (thalassemia) during hematopoiesis [1]. The hemoglobinopathies are inherited as mostly autosomal recessive traits. The reduction or absence of α-globin chains result in an excess of unpaired beta (β)-like globin chains which form insoluble homotetramers leading to intracellular precipitation, ineffective erythropoiesis and acute hemolytic anemia typical for the severe forms of α-thalassemia [2,3].

Thalassemia patient studies have played a crucial role in the identification of numerous causative mutations in the globin genes, the upstream or downstream untranslated regions, and the regulatory elements controlling the expression of the α- and β-globin gene families and thus the hemoglobin switch. Cell lines and mouse models have enabled the identification of a diverse collection of cooperating transcription factors and other protein complexes involved in the regulation of expression of these genes [4–6]. In spite of many animal models described to date, investigation of naturally occurring deletions and point mutations in carriers and patients is still essential to gain insight into regulation of expression and disease mechanisms. In the present review we'll highlight aspects of the molecular basis of α-thalassemia that provide insight into how we may understand the mechanisms underlying α-thalassemia [7,8].

2. Disease names and diagnosis

Alpha-thalassemia is characterized by a deficit in the production of the α-globin chains of hemoglobin. Individuals who carry a mutation affecting α-globin genes on a single chromosome, associated with mild anemia are said to have 'silent' α-thalassemia (if one gene is involved) or α-thalassemia trait (when two genes are involved), while compound heterozygotes or homozygotes expressing moderate to severe haemolytic anemia are known as having HbH disease. The excess of β-like globin chains form non-functional β chain tetramers called HbH (β_4 tetramers) in adults and γ chain tetramers called Hb Bart's (γ_4 tetramers) in the foetal period. The most severe form of α-thalassemia is a condition with no expression of α-genes and is called the Hb Bart's Hydrops Foetalis Syndrome (Fig. 2).

A rare syndrome, referred to as α-thalassemia/mental retardation syndrome of chromosome 16 (ATR16), is associated with very large deletions in 16p13.3 removing the α-globin genes and many other genes in and around the α-globin gene cluster (ATR16 syndrome,

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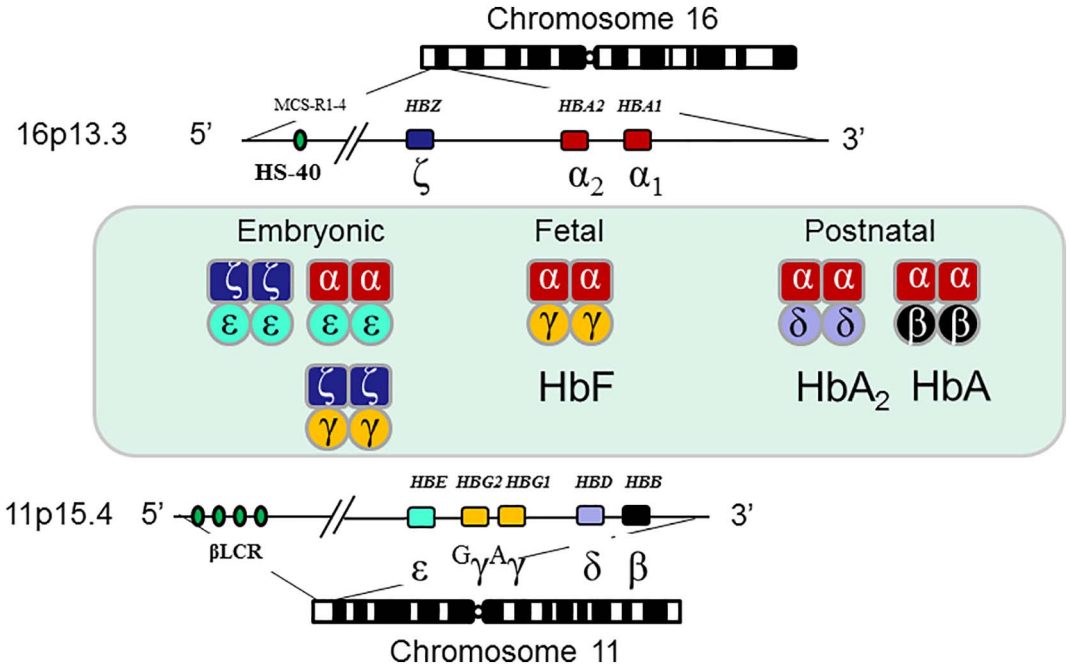

Fig. 1. Schematic presentation of the chromosomal location of the α- and β-globin gene clusters on 16p and 11p respectively. The embryonic and foetal genes are indicated as open boxes. The genes which remain active throughout postnatal life in grey and black. The different hemoglobins expressed during the embryonic period are shown, from left to right Hb Gower-1 (ζ2ε2), Hb Gower-2 (α2ε2) and Hb Portland (ζ2γ2), foetal period (HbF) and postnatal period (HbA and HbA_2).

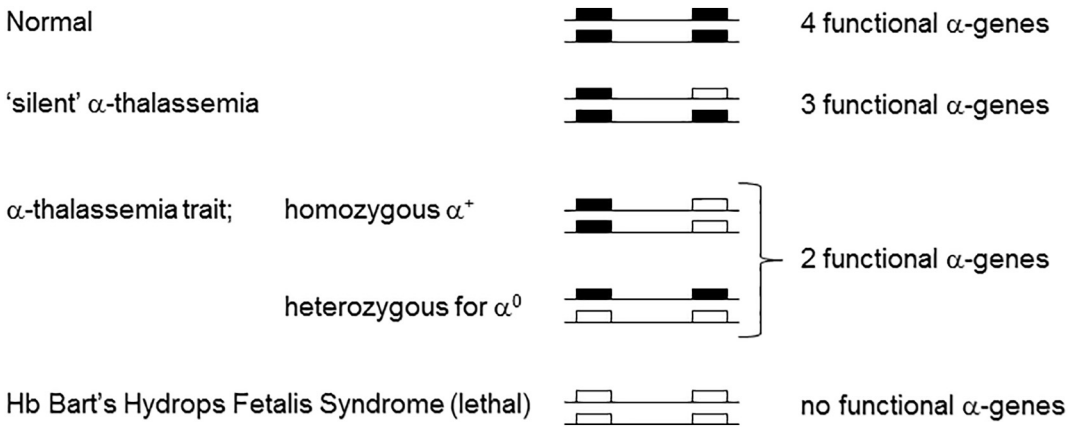

Fig. 2. Classification of α-thalassemia defects and phenotypic expression.

OMIM:141750) [9,10]. Besides marked microcytic hypochromic anemia, these patients present with a variable clinical phenotype characterized by mild to moderate intellectual disabilities, developmental delay and a wide range of less characteristic dysmorphic features. Patient analysis revealed that from the approximately 40 patients analysed to date, only 11 showed pure monosomy for the short arm of chromosome 16. The *SOX*-8 gene, strongly expressed in brain, was considered a strong candidate gene [11], however the description of a Brazilian HbH family showed none of the typical *ATR*-16 clinical features in spite of the loss of *SOX*-8 [12]. The outcome from these patient studies was that the clinical variation was so large that it could not be attributed to a single gene missing in the deleted area. One possibility is that the deletion of a large number of genes may unmask mutations normally compensated for by genes in its homolog. Another explanation could be that dosage sensitive genes may be present in the deleted area. In spite of the fact that only few *ATR*-16 patients could be investigated, imprinting doesn't seem to play a role as no major clinical differences have been seen between maternally and paternally derived chromosomes. The *ATR*-16 syndromes and the deletions involved have been extensively reviewed elsewhere [12–15].

Another rare syndrome associated with α-thalassemia is the X-linked mental retardation syndrome ATR-X. This syndrome is characterized by severe mental retardation and dysmorphic features showing striking similarities among patients. The mental retardation is more severe than in ATR-16 and involves some other clinical features such as severe psychomotor impairment, hypertelorism and facial dysmorphism like flat nasal bridge, triangular upturned nose, wide mouth and developmental abnormalities in genital and/or urogenital areas [16]. The molecular cause of ATR-X is mainly from point mutations in the *ATRX* gene (Xq13.3) encoding a chromatin-associated protein belonging to the SNF2 family of helicase/adenosine triphosphatases (ATRX OMIM:301040, reviewed elsewhere) [16].

Although the relationship between ATRX mutations and α-thalassemia is not completely clear, ATRX protein was found to be a transcriptional regulator affecting α-globin gene expression [17–19]. Furthermore, the presence in rbc's of HbH inclusion bodies, that contain insoluble β4 tetramers visible as inclusions after staining with 1% Brilliant Cresyl Blue, in some patients with ATRX syndrome implies the down-regulation of α-globin gene expression. On the other hand, not all patients present with HbH inclusions. As the ATP-dependent chromatin-

remodeling factor ATRX was found to be involved in a wide variety of nuclear processes, including gene expression, DNA repair, replication and recombination it may be concluded that other genetic factors may modify the influence of ATRX mutations on α-globin chain synthesis [16,20]. Perturbation of ATRX function in model organisms leads to defective sister chromatid cohesion and congression, telomere dysfunction, aberrant patterns of methylation and ATRX plays a role in a telomerase-independent pathway of telomere maintenance abbreviated as ALT-pathway (Alternative Lengthening of Telomeres). ATRX has a strong preference for binding to repetitive heterochromatic regions such as tandem repetitive DNA several kilobases upstream of the α-globin genes in a region denoted ψζ VNTR. The size of the ψζ tandem repeat varies between patients and the degree of thalassemia is proportional to the repeat length explaining the variation in disease severity in patients with the same ATRX mutation [21,22].

Besides these two rare forms of α-thalassemia, there is an acquired form of reduced α-globin gene expression causing HbH disease in individuals with myelodysplasia, who were previously hematologically normal. The disease called α-thalassemia myelodysplastic syndrome or ATMDS (OMIM:300448) predominantly occurs in elderly males with a pre-malignant clonal hematopoietic disease. Most cases of ATMDS have acquired mutations in the *ATRX* gene in the same functional domains of the protein as seen in the congenital form called the ATRX syndrome [18,23–25].

3. Epidemiology

Like sickle cell and β-thalassemia trait, α-thalassemia is predominantly found in tropical and sub-tropical areas, where the carrier frequency may be as high as 80–90% of the population [26–28]. Even though the mechanism is still unknown, it is believed that, like the other hemoglobin related disorders, α-thalassemia is selected because carriers are better protected against the ravages of malaria falciparum. Interacting combinations of sickle cell, β-thalassemia and α-thalassemia traits are often seen in individuals from areas which have been endemic for malaria for centuries. The clinically severe forms such as HbH disease and Hb Bart's Hydrops Foetalis are predominantly seen in South East Asia, the Mediterranean Area and the Middle East. Due to migration of populations, the endemicity of thalassemia has changed dramatically over the last thirty years. Regions like North Europe and North America, previously known as non-endemic for hemoglobinopathies now face a major challenge in the diagnosis and treatment of thalassemia and sickle cell in general, and α-thalassemia in particular because of its abundance and complexity.

4. Clinical description

The clinical presentations of α-thalassemia include four widely diverse conditions from the silent carrier state, the α-thalassemia trait, the clinically variable hemoglobin H (HbH) disease to the lethal hemoglobin Bart's Hydrops Fetalis Syndrome [1] (Fig. 2). Individuals affected with α-thalassemia may have a variable hemoglobin level from normal to severe anemia (Hb 15.5–7.5 g/dl), reduced mean corpuscular volume (MCV < 79 fl)), mean corpuscular hemoglobin (MCH < 27 pg), a normal to increased red blood cell count (RBC 6–7 × 10^{12}/l) and a normal to slightly decreased HbA_2 percentage (normal subjects are usually between 2.0 and 3.3%), depending on the number of non-functional α-globin genes. During the foetal period, when the α-globin chain synthesis falls below approximately 70% of normal, the excess of γ-globin chains form Hb Bart's. The Hb Bart's peak is detected and quantified by HPLC or Capillary Electrophoresis during newborn screening to identify infants with HbH disease. An adult with HbH disease shows HbH, visible as inclusion bodies as a minor peak on HPLC or Capillary Electrophoresis during routine Hb analysis. For definitive diagnosis of α-thalassemia and in order to fully understand the clinical phenotype, molecular analysis of the α-globin gene cluster is required [29].

4.1. Alpha-thalassemia trait

Carriers of α-thalassemia are clinically asymptomatic and identified only by chance after routine hematological analyses or during antenatal screening. Usually there is no profound anemia, fatigue or other anemia related complaints, as most carriers of a single α-globin gene defect have normal hemoglobin due to a compensating increase in number of microcytic red blood cells. As carriers of mild α-thalassemia defects often have borderline hematological values, a drop in Hb level due to blood loss, bad nutrition, infection or other causes of disease may be lead to symptoms of anemia.

4.2. HbH disease

HbH disease can be caused by compound heterozygosity for two different mutations or from homozygotes for mutations as they tend to run in communities. The α-globin gene expression is reduced to less than about 30% of the normal levels. HbH disease patients have hemolytic anemia, a variable percentage of HbH, splenomegaly with sometimes hypersplenism, jaundice of variable degree besides other complications such as gallstones, leg ulcers, infections, hemolytic episodes in response to infections or therapeutics. Younger patients may have growth retardation, while elderly patients may have iron overload to intrinsic iron storage as a consequence of chronic hemolysis [1].

4.3. Hb Bart's Hydrops Foetalis Syndrome

If no α-globin is produced, infants suffer from the Hb Bart's Hydrops Foetalis Syndrome. The most common cause is the inheritance of a deletion allele having no functional α-globin genes from both parents. In some cases, a compound heterozygosity for two different defects, one severe non-deletion mutation and a non-expressing allele due to a deletion of both duplicated α-genes is responsible for a condition which lies between HbH disease and Hb Bart's Hydrops Foetalis Syndrome. These infants have variable amounts of non-functional Hb Bart's (γ4), HbH (β4) and functional Hb Portland (ζ2γ2). The clinical features are intra-uterine anemia, pronounced hepatosplenomegaly, cardiovascular deformities with signs of cardiac failure, deformities of the skeleton, retardation in brain growth and oedema. The enlargement of the foetus and the placenta due to oedema and the risk of intra-uterine death (usually between 23 and 38 weeks of gestation) and pose substantial risk factors during pregnant. Cases have been described in which the foetus was transfused *in utero* and/or shortly after birth surviving this normally lethal condition. Intensive and lifelong blood transfusion and supportive therapy are necessary but can't prevent irreversible abnormalities. The severe complications for the foetus, together with the obstetric complications are serious grounds for counselling and discussions about selective abortion [1,30].

5. Molecular basis

The α-like globin gene cluster lies in a 135–155 kb GC-rich, Alu-repeat dense and gene-dense genomic DNA region approximately 150 kb from the telomere of chromosome 16 (16p13.3). It contains three functional globin genes, *i.e.* the embryonic ζ-gene (*HBZ*) and two foetal/adult α-genes (*HBA1* and *HBA*2), three pseudogenes, *i.e.* the pseudo ζ (*HBZps*), the pseudo α1 (*HBA1ps*), and pseudo α2 (*HBM*) and the θ (*HBQ*)-gene of unknown function (Fig. 1). The α-globin gene cluster is regulated by four highly conserved noncoding sequences (MCS-R1 to MCS-R4) and several other conserved *cis*-acting regulatory sequences.

In the 1980′s and 1990′s Liebhaber et al. [31] and Molchanova et al. [32] demonstrated a difference in levels of transcription between the two duplicated α-globin genes. In foetal and adult life the steady-state

level of α2 mRNA predominates over α1 mRNA in a ratio of approximately 70% to 30%, with identical translation profiles. This predicted a dominant role for the HBA2 locus [33]. However, by studying heterozygotes for naturally occurring structural mutations of the *HBA2* or *HBA1* genes it was found that on average stable variants from the *HBA2* mutations were slightly higher than from the *HBA1* mutations, suggesting a less efficient translation of the α2 mRNA and a more equal contribution from both genes at the protein level to an approximate ratio of 60% to 40% [32,34–36]. Mutations in the dominantly expressed *HBA2* gene are expected to have a more prominent effect on the hematological parameters in carriers and patients than mutations in the *HBA1* gene.

The annotation α^+ or α^0-thalassemia is used in diagnostic practice. When the expression of a single α-globin gene is reduced or absent this is annotated as α^+-thalassemia, a patient can be heterozygous or homozygous for α^+-thalassemia. The allelic expression is reduced either due to a deletion of a single α-globin gene (deletion type of α^+-thalassemia) or a point mutation in one of the duplicated α-globin genes (non-deletion type of α^+-thalassemia).

When the expression of both α-globin genes on one allele is absent, it is called α^0-thalassemia. As these are usually deletions involving both α-globin genes, no distinction is made between deletion- or non-deletion type as for the α^+-thalassemia mutations.

5.1. Deletion type of α^+-thalassemia

The α-globin genes are duplicated and embedded into two highly homologous 4 kb units, divided into X-, Y- and *Z*- homology boxes. The most common rearrangement in this region is a single α-globin gene deletion, caused by an unequal homologous recombination during meiosis between the mis-paired X and Z-boxes giving rise to the – $\alpha^{4.2}$ and – $\alpha^{3.7}$ deletion respectively (Fig. 3a and b) [13,37]. The complementary event is an α triplication ($\alpha\alpha\alpha^{anti\ 3.7}$ and $\alpha\alpha\alpha^{anti\ 4.2}$). The deletion of a single α-globin gene is known as α^+-thalassemia deletion. The $-\alpha^{3.7}$ is the most common α^+-thalassemia deletion.

A summary of the most common α^+-thalassemia deletions is given in Fig. 4. Extensive overviews of the deletions are reported elsewhere and updated regularly [38] (http://databases.lovd.nl/shared/references/DOI:10.1038/gim.2016.218, http://globin.cse.psu.edu/hbvar/menu.html, http://www.ithanet.eu/).

5.2. Non-deletion type of α^+-thalassemia

Deletions removing one or both α-genes are far more common than point mutations or small deletions or insertions involving sequences controlling gene expression. Some of these non-deletion types of α^+-thalassemia may cause a more severe reduction in α-globin synthesis than single α-gene deletions. A wide range of mutations in the α-globin genes have been described which have an impact on expression, mRNA splicing and degradation, globin chain stability or on the interactions between the α-chain variant and the α-hemoglobin stabilizing protein (*AHSP*). Other α-globin mutations may cause interference with the α-β globin chain interaction, the formation of the heme pocket or result in an unstable variant hemoglobin or in a variant with a reduced or increased oxygen affinity (Table 1) [39].A more comprehensive lists of mutations are presented elsewhere [13] (http://www.ithanet.eu/db/ithagenes; http://globin.bx.psu.edu/hbvar/menu.html) and are updated regularly [40–42].

A special category of mutation affecting mRNA stability are the ones involving the poly-adenylation (Poly A) signal consensus sequence. Homozygosity for this type of mutations led unexpectedly to a mild HbH disease, which at the time was quite surprizing as the poly A signal

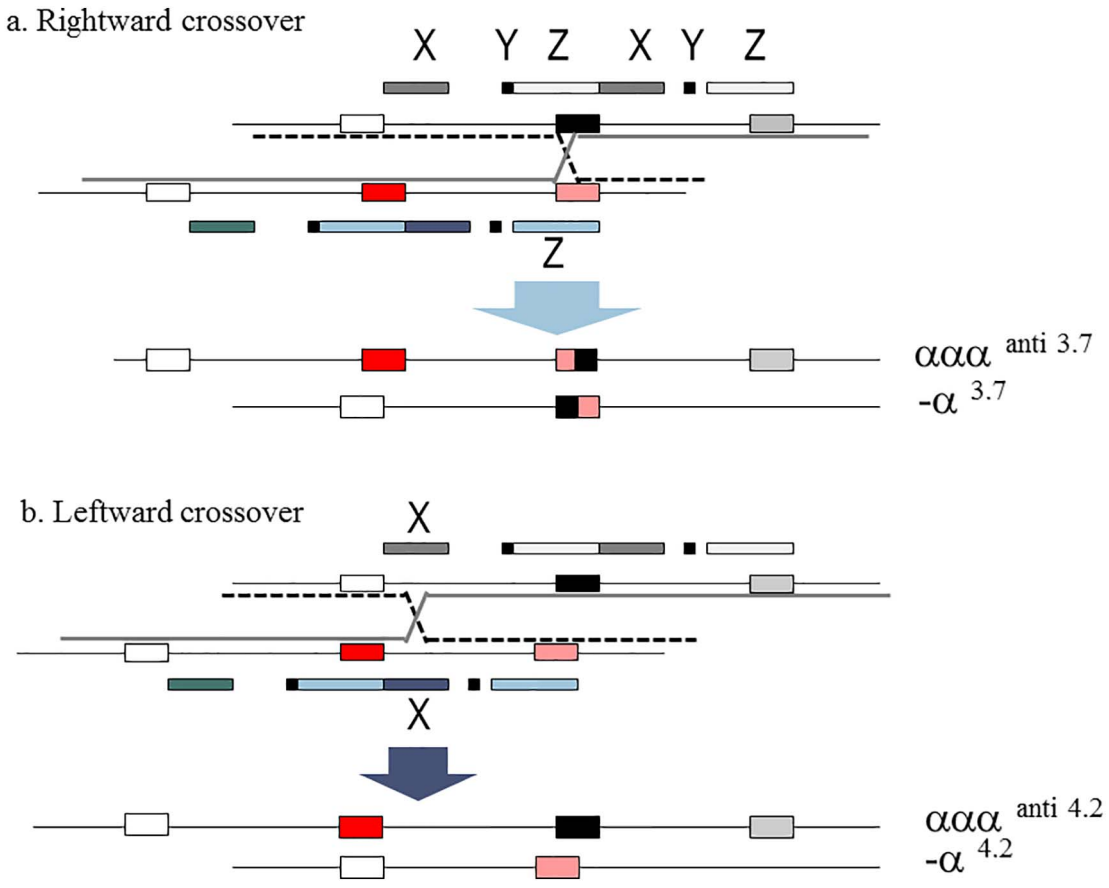

Fig. 3. Deletions that cause α^+-thalassemia. The homologous duplication units X, Y and Z in which the α-globin genes are embedded are indicated as coloured boxes. A cross-over between the mis-paired Z boxes during meiosis gives rise to (a) the $-\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\ 3.7}$ chromosomes. (b) Cross-over between misaligned X-boxes give rise to $-\alpha^{4.2}$ and $\alpha\alpha\alpha^{anti\ 4.2}$.

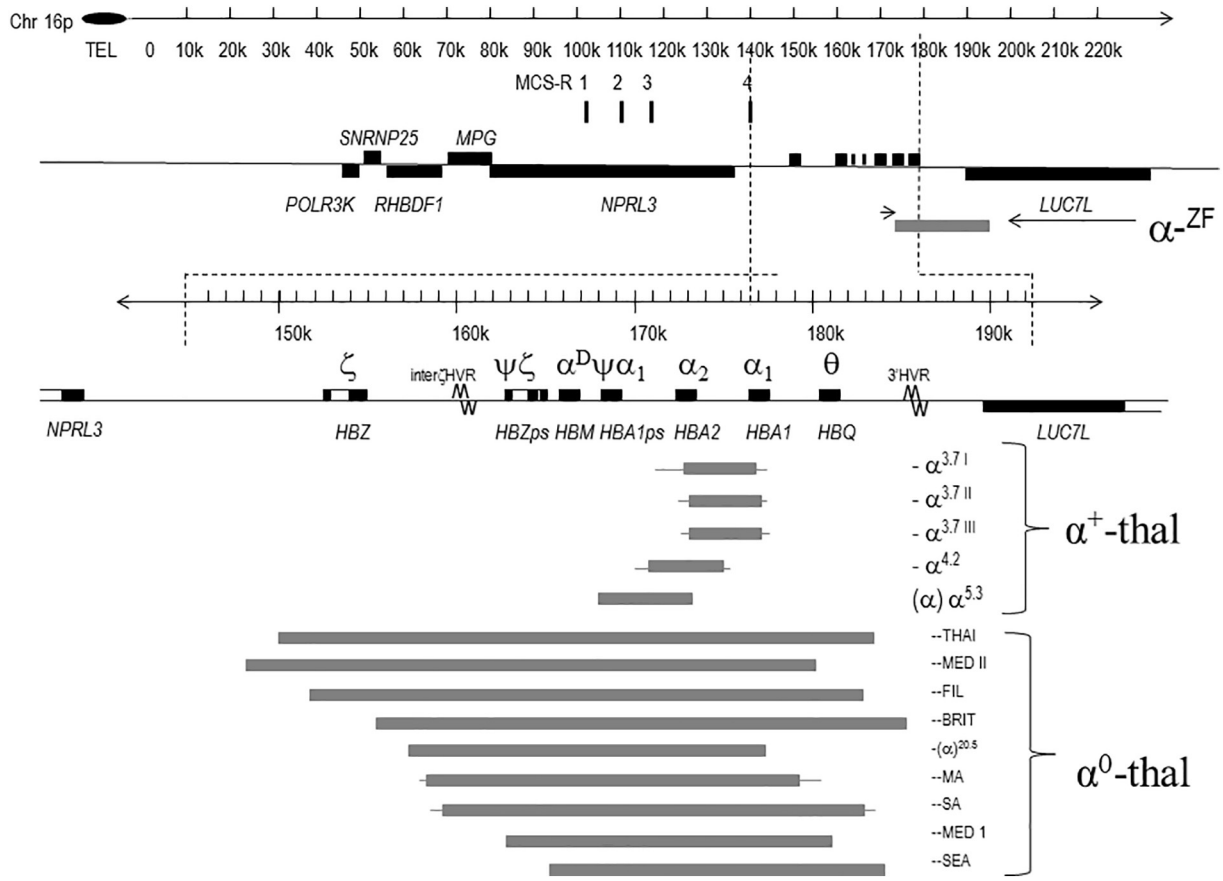

Fig. 4. Schematic presentation of the α-globin gene cluster and surrounding genes on the telomeric end of chromosome 16p. The telomere is represented as an oval, genes are shown as boxes. The α-globin regulatory region (MCS-R 1 to 4) is indicated as vertical bars. The scale is in kilobases (kb). Below the α-gene cluster is shown enlarged with the traditional and HGVS gene annotation. The most common α-thalassemia (α-thal) deletions are indicated as grey bars indicating the length of the deletion and the annotation and subdivided into α^+- and α^0-thalassemia deletions.

Table 1
Examples of non-deletional α-gene mutations and effect on expression or protein structure.

General principle of gene regulation	Type of mutation	Mechanism	Examples	Effect
mRNA processing	Donor-acceptor site mutations	Intron retention, frame shift and premature stop, reduction in mRNA output	HBA2:c.95 + 2_95 + 6delTG	α^+ or α^0-thal
	Poly A signal	Failure to terminate transcription and transcriptional interference downstream α1-gene	HBA2:c.*94A > G HBA2:c.92*A > G HBA2:c.*93_*94delAA	$\alpha^{+/0}$-thal, hom. mild HbH disease
	Cryptic splice site	Exon1 is partially spliced	HBA2:c.69C > T	α^+-thal
mRNA translation	Initiation codon	Methionine coding sequence mutated, failure to initiate translation	HBA2:c.1delA p.Met1fs HBA1:c.1A > G p.Met1Val	α^+ or α^0-thal
	Non-sense or frame shift	Premature stop codon and subsequent Nonsense Mediated Decay of mRNA	HBA1:c.44G > A p.Trp15X HBA2:c.60delG p.His21fs	α^+ or α^0-thal
	Termination codon	Elongated α-chain resulting in unstable hemoglobin tetramer	HBA2:c.427 T > C p.X143Gln (Hb Constant Spring) HBA2:c.429A > T p.X143Ley (Hb Paksé)	α^+-thal
Post translational	Missense mutations	'Silent' amino acid substitution not affecting protein function	HBA2:c.154G > A p.Gly52Ser (Hb Riccarton-II)	normal hematology, 15–25% Hb variant in carrier
		Protein instability, varying from mild to severe anemia from deficiency in α-chain synthesis	HBA1:c.43 T > G p.Trp15Gly (Hb Evanston) HBA2:c.179G > A p.Gly60Asp (Hb Adana)	α^+-thal HbH hydrops in homozygote
		Reduced AHSP binding capacity	HBA1:c.358C > T p.Pro120Ser (Hb Groene Hart)	α^+-thal
		High oxygen affinity	HBA2:c.278G > A p.Arg93Gln (Hb J-Cape Town)	Erythrocytosis
		Low oxygen affinity	HBA2:c.5 T > C p.Val2Ala (Hb Lyon-Bron) HBA2:c.175C > T p.His59Tyr (Hb M-Boston)	Mild anemia Cyanosis

mutation was found in the 3′untranslated region of *HBA2* while both alleles had intact *HBA1* genes. The genotypic effect could therefore be described as intermediate between an α^{+} and α^{0}–thalassemia allele. This suggested an effect *in cis* on the expression of the downstream *HBA1* gene. The *in vitro* analysis of the α-thalassemia *HBA2* gene with the non-functional poly A site AAUAAG transfected in HeLa cells showed that the HBA2 transcript reads through the intergenic region between *HBA2* and *HBA1* and thereby inhibiting *HBA1* gene expression [39,43,44].

Other mutations may alter the structure of the globin chain affecting the stability of the αβ-dimer or tetramer. A few examples of highly unstable Hb variants are Hb Evora, Hb Heraklion, Hb Dartmouth, Hb Quong Sze, Hb Petah Tikva, Hb Aghia Sophia, Hb Suan Dok and Hb Adana [39,45]. Such unstable proteins may precipitate causing insoluble inclusion and damage the red cell membrane. This type of α-thalassemia when transmitted through the germ line may even cause HbH-hydrops foetalis depending on which of the duplicated α-globin genes is mutated. An example of a mutation associated with different clinical effects when present on *HBA2* or *HBA1* is Hb Adana. The clinical presentation of the homozygote for the mutation on *HBA1* as present in Turks is compatible with a mild HbH disease or α-thalassemia intermedia phenotype, while homozygosity for the same mutation on *HBA2* as seen in Indonesians causes the most severe form of HbH disease, associated with HbH hydrops foetalis [46–50]. Combinations of either *HBA1* or *HBA2*-associated Hb Adana and other deletion and non-deletion α-thalassemia defects show a clinical severity varying between these two extremes [47–49,51,52].

Termination codon mutations of the *HBA2*, such as Hb Constant Spring, Hb Icaria, Hb Seal Rock, Hb Paksé and Hb Koya Dora, alter the stop codon at position 142 into a coding sequence, which results in an elongated α-chain and a highly unstable hemoglobin variant expressed at low level. The pathophysiological mechanism leading to instability and low expression is still uncertain. One possibility is the transcription of an unstable mRNA with a shortened life span, another is that the unstable Hb variant precipitates on the red cell membrane, causing hemolysis and inefficient erythropoiesis.

5.3. Deletion type of α^{0}-thalassemia

Larger deletions eliminating both duplicated α-globin genes *in cis* occur less commonly but may be present in populations in high frequency due to selective pressure by malaria tropica, which is the most deadly form of malaria caused by the parasite *plasmodium falciparum* [1,7,10,53], (Figs. 2 and 4). These deletions are called α^{0}-thalassemia deletions. If the deletion also involves the embryonic ζ-globin gene (*HBZ*), homozygotes are not likely to survive even the earliest developmental stage, however, if the deletion leaves the *HBZ* intact, homozygotes develop the Hb Bart's Hydrops Foetalis Syndrome showing continued expression of Hb Portland (ζ2γ2). Affected foetuses generally die late in pregnancy at the 23rd to 38th week of gestation. On the other hand, α^{0}-thalassemia heterozygotes even when the HBZ gene is also deleted develop normally. In addition, rare deletions of the multispecies conserved sequences MCS-R1 to R4 cause an α^{0}-thalassemia phenotype in carriers.

It is not always clear what is the mechanism involved in rearrangements in the α -globin gene cluster. Breakpoints do not always show homology such as in the common $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions, indicating that more complex recombination mechanisms may be involved. Besides the frequent involvement of Alu repeats, breakpoint analysis sometimes revealed the presence of orphan sequences originating from regions far upstream or downstream of the breakpoint. Orphan sequences are suggestive of single or multiple chromosomal loops being formed over a larger distance as intermediates of recombination leading to subsequent loss/gain of stretches of DNA being part of the loop [54,55]. Breakpoint analysis of naturally occurring human α-thalassemia deletions may help to unravel the different recombination mechanisms.

5.4. Unusual causes of α-thalassemia

Deletions in transgenic mice have shown that the MCS-R2 (previously called HS-40) is the strongest enhancer of α-gene expression and the most conserved sequence throughout evolution [10,13]. MCS-R2 and the other two critical *cis*-acting elements (MCS-R1, and MCS-R3) lie within a housekeeping gene *NPRL3*, while MCS-R4 lies upstream of the promoter of the *NPRL3* gene [7,13,20,56,57]. It has been shown that MCS-R2 is essential in chromatin looping and stable recruitment of RNA Polymerase II (PolII) to the α-globin gene promoters [53,58]. However, evidence from HbH patients homozygous for the smallest deletion (approximately 3.3 kb) known to remove MCS-R2, demonstrated that the expression of the α-globin genes was down-regulated but not completely abolished [59,60,61]. This suggests that other *cis*-acting elements are important for α-gene expression [13,62].

The mutations (point mutations or deletions) of the regulatory elements affect the recruitment of different tissue specific transcription factors (TFs) required for globin expression. Some of these TFs are KLF1, GATA-1, NF-E2, SCL and are known to play a key role in erythropoiesis [20,58,63,64]. One example of a mutation down regulating the α-globin genes by creating a new GATA-1 binding site was discovered by De Gobbi et al. [65]. In some Melanesian patients with α-thalassemia trait or HbH disease no mutations in the α-globin genes or deletions in the α-globin gene cluster were found. Extended analysis of the entire cluster revealed a small number of sequence variations including a single nucleotide polymorphism (SNP) located between *HBZ* and *HBZps* which creates a novel and functional GATA-1 binding site. The recruitment of GATA-1 to this site created by the SNP is followed by the recruitment of other erythroid TFs. This mutation creates a new promoter competing with the natural α-globin gene promoters for the remote erythroid enhancers and thus reduces the accumulation of α-globin chains.

Mutations influencing protein stability are those which affect protein-protein interactions. Alpha-Hemoglobin Stabilizing Protein (AHSP) is a chaperone that plays an important role in the stability, folding, and assembly of the α-globin subunit in the tetrameric hemoglobin molecule. A variety of unstable structural variants have been studied in carriers and patients and pointed out the role of the different amino acids in the α-globin chain in AHSP binding. The interference of ASHP binding leads to instability of the α-globin chain contributing to the pathogenicity of α-thalassemia [39]. A murine model proved that the lack of the *AHSP* gene in combination with α-globin deficiency increases the pathological conditions of α-thalassemia [66].

Sometimes naturally occurring deletions in α-thalassemia patients lead to the discovery of completely new mechanisms underlying human genetic disease. This is exemplified by the discovery of the rare > 18 kb (α^{-ZF}) deletion in an α-thalassemia family from the former Czech Republic. This deletion removes the downstream *HBA1* gene, leaving the *HBA*2 intact but unexpectedly inactive. The deletion juxtaposes the *LUC7L* gene next to the structurally normal *HBA2* gene and transcription of the unterminated antisense mRNA from *LUC7L* overlaps and interferes with normal *HBA2* expression causing an α^{0}-thalassemia allele (see Fig. 4) [61]. Since the discovery of the disease mechanism in this family, the role of antisense RNA in regulation of mammalian gene expression became eminent and a similar silencing mechanism was described later in *MSH*2 in Lynch Syndrome patients susceptible for colorectal cancer [53,67,68].

6. Genotype/phenotype correlation and diagnosis

The α-thalassemia phenotypes are classified as ‘silent’ α-thalassemia, α-thalassemia trait, HbH disease and Hb Bart's Hydrops Foetalis. Even though a broad range of clinical severity can be expected from a wide variety of interactions with Hb variants of the α- and β-

chains, β-thalassemia and multiple α-globin genes, some having an ameliorating and others a deteriorating effect on the disease phenotype. Generally speaking there is a good correlation between the clinical severity and the degree of α-globin chain deficiency on one hand and the unbalance between α- and non-α-like globin chains on the other. Non-deletion forms of α^+-thalassemia may cause a more serious reduction in α-globin chain synthesis than deletion forms, which is directly related to the clinical phenotype. Especially in the case of HbH disease, compound heterozygotes for non-deletion α^+- and deletion types of α^0-thalassemia are in general more severe than deletion types of α^+- and α^0-thalassemia.

6.1. Hematology

The red cell indices are determined by standard cell counters as present in most hematology laboratories. Various genotypes of α-thalassemia correlate well with the decrease in MCV (microcytosis), MCH (hypochromia) and Hb (anemia), and a slightly increased RBC (red blood cell count). Dedicated HPLC and CE devices are used to separate abnormal hemoglobin fractions and quantitate the percentage of (normal and abnormal) Hb fractions present. The inclusion bodies test is performed in the case of a suspected HbH disease. The typical regularly distributed stippling gives the cell a golf-ball like appearance over a blue stained background and is characteristic (Fig. 5). Carriers of α^0-thalassemia may also show an occasional HbH inclusion body in contrast to the hetero- or homozygote α^+-thalassemia carrier, which suggests that a somatic event may lead to loss of the complementary allele in hematopoietic stem cells of the α^0-thalassemia carrier giving rise to red cells deprived of α–globin. Copy-number change in the α-globin gene family has been thoroughly studied and the ectopic exchanges between homology blocks leading to the common $-\alpha^{3.7}$ deletion were also found to occur as a somatic deletion in human blood and sperm [69,70].

6.2. Alpha/beta globin chain synthesis

Only few labs are nowadays using radio-active *in vitro* synthesis of α- and β-globin chains in the patient's reticulocytes, in spite of the fact that this method is probably the most direct approach to determine the chain imbalance between the α-*versus* non-α-globin. By measuring the radioactivity of the Tritiated-Leucine incorporated into newly formed α- and β-globin chains, the ratio was determined to be around 0.75 in the case of a single α-globin gene defect, 0.5 in the case of a two α-globin gene defect and 0.25 in the case of a deficiency of three α-globin genes. First described in 1965 by Weatherall and Clegg, this method has long been used as the "gold" standard, but slowly it has been replaced by more direct and less labour intensive DNA analysis.

Additional expressed α-globin genes have been found in carriers of β-thalassemia suffering from an unexpectedly severe β-thalassemia intermedia, typically having 5 to 8 α-globin genes and only 1 functional β-globin gene. Extensive duplications of the α-globin genes including the regulatory elements have been described varying in length between 120 kb and 400 kb [71–73]. Some of the duplications were found to be arranged in a head-to-tail fashion, suggesting a back loop and strand exchange producing an extra copy of the same genomic segment during DNA replication. In contrast, the presence of extra α-globin genes with a normal β-globin genotype has no effect on the hematology of siblings in the same family. Moreover, carriers of both α^0- and β^0-thalassemia defects may have a more balanced β:α ratio similar to non-thalassemic individuals but still show a microcytic hypochromic anemia. This suggests that besides imbalance between α- and β-globin chains, also the level or dose of globin chain synthesized contributes to the thalassemic phenotype [72,74].

6.3. Molecular analysis

Over the last 40 years, DNA analysis has evolved tremendously. From Southern blotting for most arrangements commonly known today and cloning of breakpoints, to PCR amplification and direct sequencing which allows a faster characterization of breakpoints. Nowadays next generation sequencing (NGS) is applied to whole genomes searching for single point mutations, deletions, insertion and translocations [75]. However, rapid screening methods such as multiplex gap-PCR for the seven most common α-thalassemia deletions, Multiplex Ligation dependent Probe Amplification (MLPA) for less frequently occurring deletions and direct sequencing of PCR amplified α-gene fragments will still be used at large scale to increase efficiency and reduce cost. The relatively small (approximately 1.2 kb) but GC-rich and duplicated α-genes can efficiently be amplified by most routine labs using high fidelity, heat-stable DNA polymerases at specific reaction conditions using buffers containing DMSO or betaine. The most common α-thalassemia deletions are diagnosed by multiplex GAP-polymerase chain reaction using specific primers flanking the deletion breakpoints [76]. The MLPA and NGS methods are used in the diagnostic setting to detect uncommon deletions in the α-globin gene cluster [72]. Primers for sequencing, multiplex PCR and MLPA have been extensively reviewed elsewhere [77,78].As mutations causing α^+- and α^0-thalassemia may be population specific, strategic choices are made by diagnostic

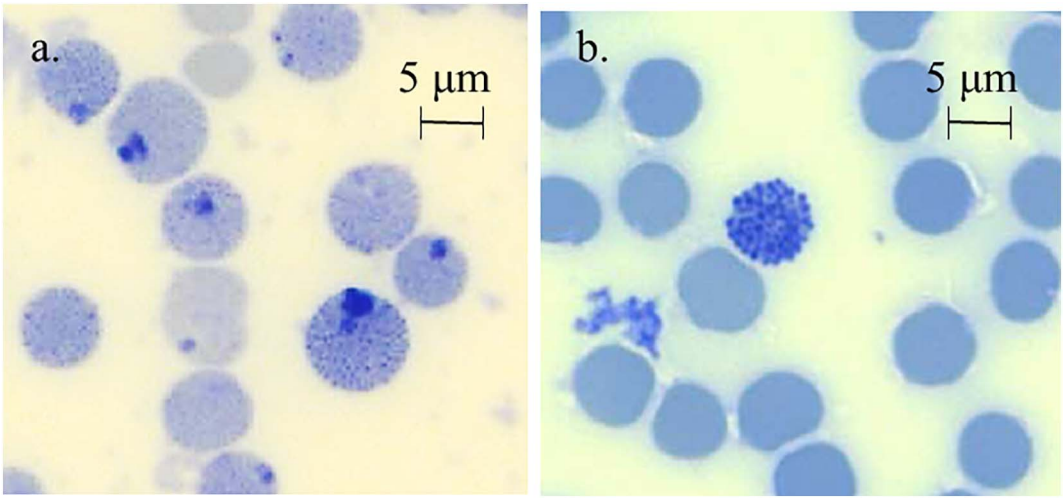

Fig. 5. Microscopic examination of the blood smear after staining with 1% Brilliant Cresyl Blue at 1000 fold magnification. (a). Red cells of HbH disease patient showing Inclusion Body positive cells (1000 × magnification). (b). Red cells of α^0-thalassemia carrier showing an occasional Inclusion Body positive cell after searching 10–20 fields (1000 × magnification).

laboratories, by selecting molecular techniques.

7. Differential diagnosis and genetic counselling

α-Thalassemia trait can be confused with iron deficiency anemia as the hematological parameters are quite similar showing decreased MCV, MCH and Hb. Therefore iron status by measuring ferritin or Zinc Protoporphyrin (ZPP, a measure for long lasting iron deficiency) should be properly assessed to distinguish between the two conditions. A distinguishing feature may also be the elevated RBC as often seen in individuals with α-thalassemia trait in contrast to the decreased RBC frequently seen in iron deficiency anemia. As carriers of α^{+}-thalassemia, in particular the $-\alpha^{3.7}$ allele, often present with normal hematology, confirmation at the DNA level is necessary for a definitive diagnosis. Interaction of deletion and non-deletion forms of α-thalassemia, α-globin gene duplications, as well as mutations at the β-globin gene (causing β-thalassemia, HbS, HbE or others), will influence the hematology and clinical expression of defects seen in patients. Therefore the correlation between hematological analysis, hemoglobin separation by HPLC and/or CE and molecular analysis of the α- and β-globin genes and gene-clusters by direct sequencing and MLPA is considered to be 'best practice' in the definitive diagnosis of hemoglobinopathies [29,78].

The main reason for genetic counselling for the α-thalassemia syndromes is the avoidance of the Hb Bart's Hydrops Foetalis Syndrome, which can result in a range of clinical issues most severely neonatal death and can also have serious health risks for mother also during pregnancy. The HbH disease is usually mild (comparable to β-thalassemia intermedia), but the clinical and hematological severity can vary considerably. HbH disease is generally not considered for prenatal diagnosis unless a previous clinical history in the family has been recorded. Even though molecular knowledge about the mutations may allow some level of predictions towards the expected disease severity this remains uncertain.

8. Management and targets for treatment

8.1. Management of α-thalassemia trait, HbH and Hb Bart's

Usually individuals with α-thalassemia trait do not need treatment. Carriers can be anemic however as a consequence of nutritional deficiencies related to hematopoiesis, such as vitamin B12, folate- or iron deficiency. Prophylactic iron suppletion should be carefully measured by checking the ferritin levels in order to prevent iron overload.

Even though HbH is generally considered to be a mild condition, the clinical course is often considered more severe than previously recognized. The deletion types of HbH disease, with a milder clinical presentation may require intermittent blood transfusion during intercurrent illness but some cases have required regular transfusion. The group of non-deletion types of HbH disease requires more regularly blood transfusions and chelation therapy. They may show marked splenomegaly that occasionally leads to splenectomy.

The way to handle the risk for Hb Bart's Hydrops Foetalis Syndrome is generally counselling and prenatal diagnosis, because of the maternal and foetal morbidity risk and ineffective therapeutic options. In a few reported cases intra-uterine transfusion has been performed following the diagnosis of an α^{0}-thalassemia homozygote, preventing hydropic features and sometimes severe neurological or congenital abnormalities, but most surviving infants have still developed limb and congenital urogenital defects [1,30,38].

8.2. Future targets for treatment

Currently the recommended curative option for β-thalassemia major and sickle cell disease (SCD) is allogenic hematopoietic stem cell transplantation (AHSCT) if an HLA compatible donor is available. For patients lacking a suitable HLA compatible donor, *ex vivo* gene therapy using autologous hematopoietic stem cells is considered an option for curative treatment. DNA-base strategies for gene-therapy are mainly focussed on the development of experimental therapeutic approaches for β-thalassemia and SCD.

Three major objectives of therapeutic interventions can be considered: firstly, the correction of the altered β-globin gene expression by gene substitution using homologous recombination, or by correcting the molecular defect using modifying oligonucleotides; secondly, elevating the HbF expression to a clinically relevant amount. This can be done by vector mediated transfer of normal γ-globin genes or by interference of transcription repressors of γ-globin gene expression, such as *BCL*11*A*, *KLF*-1 and *MYB* [79–87].

The third objective involves the down regulation of α-globin gene expression. Ineffective erythropoiesis is due to the accumulation of the free excess of α-globin in erythroid precursor cells, which is one of the key pathophysiological mechanisms in β-thalassemia. It has been known from many combinations of thalassemia mutations that the natural reduction of α-globin synthesis ameliorates the disease phenotype in β-thalassemia patients. Genome editing approaches can be directed to disrupt the α-globin gene expression directly by gene targeting, for instance by post-transcriptional silencing through RNA interference (RNAi) using small interfering or short hairpin RNAs or through targeting regulatory elements of the α-genes such as epigenetic drugs altering the chromatin environment or genome-editing to disrupt α-gene expression [88–90].

An important breakthrough in gene therapy for hemoglobinopathy came from *ex vivo* lentiviral transfer to hematopoietic stem cells curing a sickle cell patient [91]. A modified β-globin gene (β^{AT87Q}-globin) was used to inhibit HbS polymerization [87,92]. Several clinical trials of gene therapy for β-thalassemia major and sickle cell disease are ongoing [87].

Several gene editing tools have been developed over the last 10 years, the most important is CRISPR-Cas9 (Crispr-associated protein 9 nuclease) [93–95]. Induced pluripotent stem cells (iPSCs) from different cell types from β-thalassemia patients have been used to correct genes using the CRISPR-Cas9 technology [96–98]. In theory this could also be applicable for α-thalassemia by restoring the number of α-globin genes, or alternatively by reducing β-globin gene expression in an attempt to ameliorate the clinical severity of for example HbH disease. At present the safety issues concerning transplantation of iPSCs to the patient still need further study [84,99,100].

Recently, the α^{D}-globin (also known as pseudo α2-globin gene or *HBM*) has become of particular interest as evolutionary studies revealed a high homology to the avian α^{D}-globin genes. The majority of birds and nonavian reptiles co-express two functionally distinct isoforms of the hemoglobin molecule, the major adult HbA which consists of α-globin chains expressed by the α^{A}-globin gene and the minor adult HbD encoded by the α^{D}-globin gene [101,102]. The finding of a highly conserved human homolog of the α^{D}-globin gene called *HBM*, made biologists wonder if α^{D}-globin could function as a substitute for the shortage of functional α-globin in α-thalassemia syndromes [103].

The human *HBM* gene is located between *HBZps* and *HBA1ps* (Fig. 4) [104]. Even though the discovery of mRNA products in humans, pigs and cows suggests that the *HBM* gene is expressed and likely to be functional, no globin was demonstrated. In spite of an intact transcriptionally active copy in humans as well as in some other mammalian lineages of this seemingly defunct α^{D}-globin gene there is no evidence that the product is assembled into functionally intact hemoglobin tetramers at any stage of development [105,106,102,107]. Also in birds the α^{D}-globin gene has been deleted or inactivated multiple times, which may reflect the minor HbD isoform is subject to less stringent functional constraints [102,108–111]. From homozygosity of the—SEA deletion, which causes Hb Bart's Hydrops Foetalis Syndrome, it becomes clear that in the complete loss of *HBM* does not affect human embryonic development, as these foetuses survive until birth. In

addition, of the > 130 different molecular defects known to cause α-thalassemia besides an ever increasing number of interactions, no evidence was found indicating that the *HBM* gene is vital in human hematopoiesis. Whether the α^{D}–globin could be of potential interest as a target to compensate for the loss of α-globin, genes mainly depends on the possibility to translate the transcript into protein and the capacity to form tetramers with human β-globin.

9. Concluding remarks

The variation in human mutations leading to the thalassemia syndromes have taught us a lot about how the α-globin genes are regulated and about the more general concept of gene regulation in human disease. Although the molecular basis of α-thalassemia has been extensively studied, the importance of genetic variability between individuals remains unclear. The molecular mechanisms leading to chromosomal rearrangements, telomere truncations, homologous recombination, gene conversion and copy number variation are of particular importance in the modulation of thalassemia disease severity. Furthermore, changes in tandem repeats, abnormal methylation, the involvement of antisense RNA are identified as mechanisms in globin gene disorders that need to be clarified further. Studies on a few rare cases carrying deletions within regulatory elements but presenting a phenotype less severe than expected demonstrate other possible genetic and/or epigenetic regulation mechanisms of the human *α*-globin genes [58–60].

Moreover, the limitations of the *in silico* software as the first means to predict the potential deleterious effect of a nucleotide substitution, should be taken into account as they only consider the 3D structure of a protein but not for instance, intronic changes [112]. Thus, it is possible that they underestimate the significance of the changes that may lead to globin instability due to intermolecular interactions in Hb tetramer, either interactions with other proteins such as AHSP or a possible posttranslational modification. The presence of some assumed neutral SNPs in unexpected α-thalassemia phenotypes shows the possible impact of these nucleotide changes on α-globin gene function or stability [113,114].

As we learn from the variety of human mutations, it needs to be remembered that all regulatory elements and functional genes were discovered by looking at naturally occurring deletions and point mutations in human patients and carriers. The continuous search for novel mutations and interactions of both deletions and non-deletion variants in patients with an unexplained severe or mild phenotype will continue to increase our knowledge of globin-gene regulation and expression and will lead to new targets of therapy.

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