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ORIGINAL ARTICLE

The Chaperones Involved in Hemoglobin Synthesis Take the Spotlight: Analysis of *AHSP* in the Argentinean Population and Review of the Literature

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ABSTRACT

Hemoglobin (Hb) synthesis is a complex, well-coordinated process that requires molecular chaperones. These intervene in different steps: regulating epigenetic mechanisms necessary for the adequate expression of the α- and β-globin clusters, binding the nascent peptides and helping them acquire their native structure, preventing oxidative damage by free globin chains and preventing the cleavage of essential erythroid transcription factors. This study analyzed the distribution of the single nucleotide polymorphism (SNP) *rs4296276* in intron 1 of the α-globin chaperone α Hb-stabilizing protein (AHSP) in the Argentinean population. The risk allele was found in thalassemia patients who exhibited more severe phenotypes than expected. Future studies may help establish the role of these chaperones as modifiers in pathological states with globin chain imbalance, such as thalassemia.

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Introduction

Hemoglobin (Hb) synthesis is a complex process requiring, on the one hand, that the erythroid precursors express different genes from the globin families in precise moments of development of an individual, and, on the other hand, an exquisite coordination of the expression of the β- and α-globin *loci*. The first process is known as the 'globin switch.' Two switching events take place in the β-globin family: the first one, when the fetal erythropoiesis migrates from the yolk sac to the liver and marks the beginning of the expression of the *HBG2* and *HBG1* genes, while the *HBE1* genes ceases its expression, whereas the second one occurs about 6 months after birth, when the *HBB* gene becomes the main gene transcribed [1]. Only one switching event takes place in the α-globin family: around weeks 6–7, the expression of the *HBZ* gene is turned out, and the *HBA2* and *HBA1* genes gradually become the only genes from this cluster translated in the fetal and postnatal periods [2,3]. These processes are strictly controlled by general and essential erythroid specific transcription factors, such as GATA binding protein 1 (GATA1) and Krüppel-like factor 1 (KLF1) [4].

This study presents the different chaperones involved on the process to obtain functional adult Hb focusing in how some of these chaperone complexes operate under the stress of pathological states such as β-thalassemia (β-thal). Both globin gene families require the deposition of H3.3 for their efficient transcription, though they differ in the chaperone machinery that assists this process. The histone cell cycle regulator (HIRA) complex, composed of HIRA, ubinuclein-1 (UBN1) and calcineurin binding protein 1 (CABIN1), with the concerted action of anti-silencing function 1 A histone chaperone (ASF1a) [5], regulates transcription at the β-globin cluster and has been implicated in erythroid differentiation and maturation: an ablation of the HIRA protein led to a deficiency of the erythroid essential factors GATA1 and KLF1. In turn, KLF1, through its zinc finger domain, interacts with the carboxy-terminus region of HIRA, and enables its selective recruitment to the promoter of some of its target genes, such as *HBB*. KLF1 is crucial in establishing the correct 3D structure at the β-globin locus and opening of the chromatin, and it was established that the recruitment of HIRA by KLF1 leads to an enrichment of H3.3 in the β-globin promoter [6].

The deposition of H3.3 in the α-globin *locus* is mediated by the complex formed by the chromatin remodeler X-linked α-thalassemia (α-thal)/mental retardation (ATRX) and the chaperone death-associated protein 6 (DAXX). As previously described [7–9], mutations in *ATRX* lead to α-thal with intellectual disabilities and developmental defects, such as genital abnormalities, microcephaly, hypertelorism, epicanthus, a small triangular upturned nose, and flat face.

The ATRX-DAXX complex, through H3.3 deposition, maintains the boundary between regions of transcriptionally active and inactive chromatin and the loss of ATRX may result in the spreading of heterochromatin along DNA, with the progressive downregulation of nearby genes in *cis*, such as the α-globin family [10]. It was also hypothesized that ATRX recognizes nucleosomes that present H3K9me3 and participates, by interacting with DAXX, in the exchange of macroH2A for H3.3 at the α-globin *locus* [11].

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Table 1. Distribution of the *rs4296276* genotypes in the β-thalassemia groups and control population.

Parameters	G/G	A/G	A/A
Severe β-thal carriers	3	3	0
β-TI	30	3	1
β-TM	11	5	0
Control population	55	19	7

β-TI: β-thal intermedia; β-TM: β-thal major.

Regarding the folding of the globin chains, it was already elucidated that the erythroid precursors express a private α-globin chaperone, α hemoglobin-stabilizing protein (AHSP), which helps with the folding of the nascent α-globin chains, the incorporation into αβ tetramers, the refolding of denatured α-globin and contributes to detoxify excess α-globin [12]. This 11.84 kDa protein is mainly expressed in hematopoietic tissues, by cells of the erythroid lineage [13] and correlates suitably and stoichiometrically with α-globin expression, reaching a peak, during erythroid differentiation at the polychromatic and orthochromatic stages [14]. The *AHSP* gene maps in chromosome 16p11.2, and its expression is governed by the erythroid transcription factors GATA1 and KLF1 [15] and the universal factor class 2 homeobox 1 (POU2F1; also known as OCT1). It is also regulated by the iron status [16]. It was a recently described role of AHSP as a chaperone for the arteriolar endothelial cell-expressed α-globin, which plays an important role in lessening the vasodilatory affects of nitric oxide (NO) on the nearby vascular smooth muscle. It plays a dual role, both promoting expressed α-globin proper expression and facilitatating the (Fe^{3+}) α-globin reduction by the endothelial NO synthase (eNOS) [17,18].

Upon conditions with an excess of free α chains, 70 kDa heat shock proteins (Hsp70) may act as a complementary chaperone to prevent an excess of free globin peptides. Hsp70 helps the progression of erythroid differentiation, as it protects GATA1 from caspase 3, by binding this transcription factor in the nucleus, thus avoiding apoptosis [19]. It was observed in β-thal major (β-TM) erythroblasts, that Hsp70 binds directly to the free apo-α-globin chains. Therefore, it does not translocate to the nucleus and does not bind GATA1, resulting in end-stage maturation arrest of polychromatophilic erythroblasts and apoptosis [20].

Until recently, there were no reports of chaperones involved in the folding of β-globin chains. However, Ghosh *et al.* [21] reported the crucial role that 90 kDa heat shock proteins (Hsp90) plays in erythropoiesis by acting as a chaperone for the apo-β-globin-like peptides (β and γ chains were evaluated): it stabilizes the apo-globins, helps to drive their heme insertion reactions and then dissociates to allow the formation of the heterotetramers with the α-globin chains. Furthermore, in tissues where the globin genes are expressed but the AHSP chaperone is not, Hsp90 is able to bind the apo-α-globin chains, supporting the maturation of both α- and β-globin. Hsp90 also contributes to Hb synthesis by acting as a chaperone for the nascent heme regulated inhibitor of translation (HRI) [12].

Out of these chaperones, AHSP has been the most extensively studied as a candidate modifier in thalassemia. A murine model of β-thal intermedia (β-TI), showed worsened symptoms upon AHSP loss [22] and Galanello *et al.* [23] reported the association of reduced AHSP mRNA levels and a more severe phenotype in individuals with the same β-thal genotypes in two unrelated Sardinian families. However, other reports showed that mutations affecting AHSP are infrequent, disregarding this chaperone as a disease modifier [24]. Nevertheless, variants that affect the activity of AHSP, such as p.N75I, that impairs its role in inhibiting the production of reactive oxygen species, showed clinical consequences when associated with the β-thalassemic mutation codon 39 (*HBB*: c.118C>T) [25]. In the same study, two non coding variants were associated with variation of AHSP expression: a variable length T-homopolymer in the proximal promoter region (the T15 allele showed reduced mRNA expression in reticulocytes, compared to the T18 allele) and a single nucleotide polymorphism (SNP) (*rs4296276*) (NM_001318222.1:c.-4-27G > A) in the first intron, which disrupts the binding of POU2F1, as shown in *vitro*.

In contrast, variants that impair the formation of the α-globin/AHSP heterodimers could lead to α-thalassemic syndromes. However, it is difficult to evaluate the impact of mutations affecting this interface, as most variants impair the binding of the β-globin chain as well. Substitutions of Lys99 of the α-globin result in attenuated binding to AHSP without affecting the β-globin interaction, and impair protein folding and expression in *vitro* with a mildly destabilizing effect in *vivo* [26]. On the other hand, the mutation affecting AHSP, p.V56G, detected in a homozygous state, caused an α-thal-like syndrome in a child with no mutations on the α-globin cluster [27]. This study analyzed the frequency of the SNP *rs4296276* in the Argentinean population and its distribution in patients with thalassemia, considering a candidate phenotype modifier.

Patients and methods

Genomic DNA of 137 subjects (132 unrelated) was isolated from peripheral blood leucocytes using the standard cetyltrimetilammonium bromide (CTAB) method [28]. Fifty samples were from patients diagnosed with clinically relevant forms of β-thal [34 β-TI; 16 β-TM], six carriers of severe β-thal, as defined by their physicians, while the remaining 81 samples were obtained from hematologically normal controls. Written informed consent, following the current version of the Helsinki Declaration, was obtained from the individuals involved in this study, and the research project was approved by the institutional bioethics committee.

The sequence variant *rs4296276* in intron 1 of *AHSP* was determined by allele-specific polymerase chain react (PCR) with primers designed with the online software WASP [29] (rs4296276-F-A: 5′-AGT GAG GAG GGA AAC AGA TAG A-3′; rs4296276-F-G: 5′-AGT GAG GAG GGA AAC AGA TAG G-3′; rs4296276-R: 5′-GCT CCA ATT ATC CTC TTC CT-3′). The PCR product of 209 bp maps from 31,446,910 to 31,447,118 on chromosome 16. The statistical analyses were performed with GraphPad Prism 5 (GraphPhad Prism Inc., La Jolla, CA, USA).

Results

Of 132 unrelated individuals, 94 were homozygous for the G allele, 30 were heterozygotes and eight presented the A allele

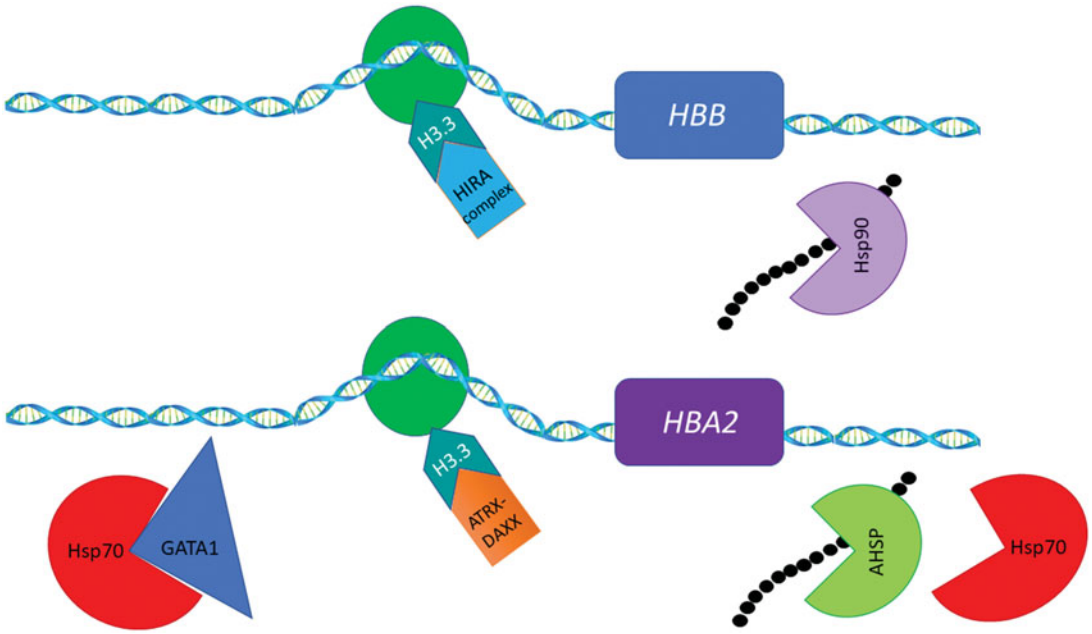

Figure 1. The chaperones involved in Hb synthesis. The HIRA complex and ATRX-DAXX are involved in the deposition of histone variant H3.3 in the regulatory regions of the β- and α-globin families, respectively; ATRX also keeps the variant macroH2A away from the α-globin cluster. Hsp90 acts as a chaperone for the nascent β-globin-like chains, whereas the α-globin peptides have their own private chaperone, AHSP. In physiological states, Hsp70 binds the mature GATA1 factor, preventing its degradation by caspases. When there is an excess of free α-globin chains, these are also bound by Hsp70, inhibiting the interaction with GATA1.

in a homozygous state. This distribution of genotypes implied that the population does not meet the Hardy-Weinberg assumptions (χ^2 test p value = 0.015729). The minor allele frequency was 0.17.

As the population was not in Hardy-Weinberg equilibrium, it was not advisable to perform association studies. However, it was interesting to analyze if the allele frequencies varied in the different groups of patients and controls. Fisher's exact tests were performed to compare the distribution of the A (risk) and G (protection) alleles between the β-thal patients and controls ($p = 0.1340$) and between the patients with β-TI and β-TM ($p = 0.2764$). No significant differences were found between the groups. The distribution of the genotypes is shown in Table 1.

Discussion

Emerging data is revealing a more prominent role of the molecular chaperones in the proper expression of the globin chains (Figure 1). However, more studies are required to evaluate how differences in the expression or structure of these chaperones can impact Hb synthesis.

It is remarkable the importance of the H3.3 histone variant for the adequate expression of both α- and β-globin gene families; the correct deposition of this variant by DAXX-ATRX with the exclusion of macro2HA is crucial for the expression of the α-globin genes and the interaction of the HIRA complex and H3.3 is necessary for the expression of the essential erythroid factors GATA1 and KLF1 and for the second switch in the β-globin cluster, leading to the transcription of the *HBB* gene.

Regarding the chaperones that can bind directly the nascent as well as denatured globin chains (AHSP, Hsp70 and Hsp90), it is interesting to entertain the possibility that at least one of these molecules might play a role in modulating thalassemic phenotypes. Although there is some clinical evidence of the impact of AHSP differential expression in patients with β-thal [23], sequence variants affecting this gene with proven effect on the phenotype, are not very frequent. However, it may be worth studying in these patients the SNP *rs429627*, which, at least in *vitro*, impacts the synthesis of ASHP by disrupting the binding site of the transcription factor POU2F1.

According to the reports in the gnomAD database (http://gnomad.broadinstitute.org/), this SNP presents a frequency of 0.1978 (55,521 alleles out of 281,952) and was present in every population analyzed. In our population, the frequency of the 'A' allele was 0.17. The fact that this variant presents a frequency of less than 1.0%, reinforces the possible deleterious nature of the minor allele.

It is remarkable that the 'A' variant at the heterozygous state was found in three severe β-thal carriers who only carry a β^0 or β^+ severe mutation (*HBB*: c.92 + 1G>A in two cases and *HBB*: c.93-21G>A the remaining one) and presented no copy number alterations on the α-globin cluster. It is possible that in this group of patients, who already exhibit a mild α/β chain imbalance, a diminished expression of the AHSP chaperone could increase the pool of free α-globin chains, at least slightly, worsening the phenotype.

It is likely that no significant differences between the genotypes of the thalassemia patients and the control population can be determined, as, in conditions of equimolar production of α- and β-globin chains, the effects of this polymorphism are not evident. However, it is still possible for this variant to modulate the phenotype. Interestingly, one of the β-TM patients who presented the A/G genotype also exhibited the mutations *HBB*: c.-137C>G and *HBB*: c.93-21G>A as primary modifiers, which are compatible with a

β-TI phenotype. The 'A' allele, also in a homozygous state, was found in a patient with the *HBB*: c.118C>T β-thal mutation and a duplication of the α-globin cluster (Supplementary Table 1).

Although the Hsp70 and Hsp90 complexes could be induced by the hematopoietic stress in severe forms of thalassemia, there are no reports that show a differential expression of these chaperones; a study in both early and late erythroid progenitor cells from healthy donors and patients showed no statistical difference in the expression of the genes that encode these proteins [30]. It is noteworthy that the cell has redundant molecules capable of binding free α-globin chains, in order to limit their toxicity; AHSP is the usual partner, but Hsp70 binds these peptides under certain conditions and Hsp90 is also capable of binding them in tissues that do not express AHSP.

Until recently, there was no evidence that the nascent β-globin-like peptides needed help to acquire their native state. The fact that the Hsp90 chaperone system is involved, can lead to new studies to understand the extent of its role. It is especially promising for dominant thalassemia due to hyperunstable β-globin chains. More in-depth studies will be needed to understand the nature of this interaction, in order to provide alternative mechanisms to facilitate the binding of the altered peptides to the chaperone system and lead to its proteolysis by the ubiquitin-ligase system. In conclusion, guarding Hb synthesis is a complex, geared-well process that requires the collaboration of all these underrated helpers. Future studies will determine the length of the impact they exert.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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