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Alpha hemoglobin stabilizing protein: Its causal relationship with the severity of beta thalassemia

Chandan S. Sagar [a], Rakesh Kumar [a], Dharmesh C. Sharma [b], Purnima Kishor [a,*]

[a] School of Studies in Biochemistry, Jiwaji University, Gwalior, India
[b] Blood Bank, Jaya Arogya Hospital, Gajra Raja Medical College, Gwalior, India

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ABSTRACT

Thalassemia major is characterized by anemia, iron overload and cellular damage. The severity of symptoms correlates with the alpha/non-alpha globin imbalance and is proportional to the magnitude of alpha chain excess. Alpha hemoglobin stabilizing protein (AHSP), the erythroid specific alpha globin chaperone, stabilizes free alpha chains, and prevents the formation of reactive oxygen radicals. Though AHSP expression has been linked to the severity of beta thalassemia, its role as a probable genetic modifier of disease severity, has still not been unequivocally established. In the present study, the level of the chaperone has been seen to vary in regularly transfused beta thalassemia patients, being underexpressed in 64% of cases, upregulated in 16% and comparable to controls in 20% of the cases. This discrepancy may be attributed to the degree of DNA damage, % HbF, and the number of nucleated RBCs in the peripheral blood of these patients. Results reveal that a decrease in the free alpha chain pool, and hence the repertoire of unbound iron, due to elevated HbF and/or the presence of nucleated RBCs in the peripheral blood results in the upregulation of the AHSP gene.

1. Introduction

Excess alpha hemoglobin is cytotoxic and is largely responsible for the pathophysiology of beta thalassemia. Alpha-hemoglobin-stabilizing protein (AHSP), the erythroid specific molecular chaperone for free alpha globins, stabilizes and prevents the precipitation of alpha chains and might be important in β-thalassemic erythropoiesis characterized by an imbalance of globin chain synthesis [1]. In humans, AHSP expression levels vary among different individuals because of polymorphisms in regulatory regions and perhaps additional determinants that are not linked to the AHSP gene [2,3]. There is uncertainty regarding the direct relationship between AHSP expression levels and beta thalassemia phenotype. Some studies indicate that AHSP expression levels inversely correlate with the severity of thalassemia [2] while in others, disease severity was not found to correlate with the gene haplotypes [4,5].

The loss of AHSP function in mice model resulted in abnormal erythrocyte morphology, which shows cellular damage due to increased ROS and intracellular inclusion bodies. This results in an increased destruction in erythroid precursor cells and a reduced lifespan of erythrocytes [6]. These phenotypes could be the result of the loss of AHSP, causing nascent α-globin to be structurally unstable, making it incompatible for HbA formation [7]. Lim et al. [7] have reported significant correlation between AHSP expression and mean cell hemoglobin, HbF %, α-globin, β-globin and excess α-globin expression and conclude that AHSP could be a secondary compensatory mechanism in red blood cells to counterbalance the excess α-globin chains in HbE/β-thalassemia individuals.

In the present study the level of AHSP in beta thalassemia patients as compared to non-thalassemic controls has been determined and its relation with DNA damage, ineffective erythropoiesis and HbF has been demonstrated in order to provide an insight into the causal relationship between the level of AHSP and the severity of beta thalassemia.

2. Methods

2.1. Subjects

Subjects included 30 regularly transfused beta thalassemia patients of the Gwalior Chambal region, registered at the blood bank of Jaya Arogya Hospital, Gajra Raja Medical College, Gwalior. The age of the patients ranges from 2–19 years, the average age being 10 years. Five patients were β° heterozygotes and the rest were homozygous for the severe β + IVS-1-5 (G>C) [HGVS nomenclature c.92+5G>C] mutation of the beta globin gene. Blood drawn for cross matching, prior to transfusion, was taken for analysis. Written informed consent was taken from the parents/guardians of all the patients prior to the analysis. Blood samples of non-thalassemic adult volunteers served as controls.

* Corresponding author.
E-mail address: poornima1kishore@yahoo.com (P. Kishor).

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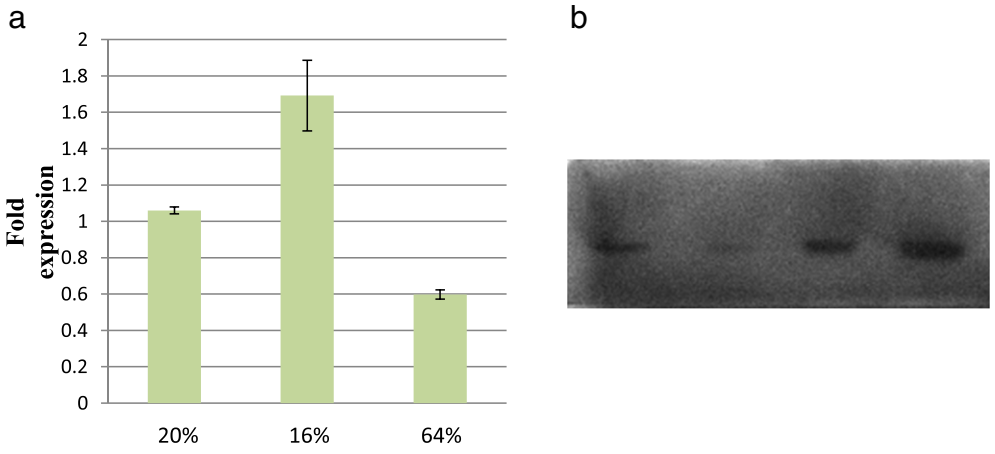

Fig. 1. a) Fold expression of AHSP in % cases. b) Western blot: Lane 1_ AHSP in non-thalassemic control, lanes 2_5 AHSP in beta thalassemia patients.

2.2. Determination of HbF

HbF was determined by cation exchange high performance liquid chromatography by the HbA2/HbF/HbA1c dual program of the D-10 HPLC system of Bio-Rad, Laboratories.

2.3. Nucleated RBCCs

Whole blood was stained with Giemsa stain and observed at 100× under the microscope and nucleated RBC counts were determined per 100 RBCs.

2.4. Quantitation of AHSP

Relative quantitation of AHSP of beta thalassemia patients as compared to non-thalassemic controls was done by Western blotting [8].

2.4.1. Sample preparation

RBCs were separated by layering whole blood on Hi Sep (Hi Media), lysed, mixed with sample buffer (10% w/v SDS, 10 m M dithiothreitol, 20% v/v glycerol, 0.2 M Tris HCl, pH 6.8, 0.05% bromophenol blue), boiled in a water bath at 60 °C and loaded. Samples were normalized to the micrograms of protein loaded. Protein was determined by the Lowry's method [9].

2.4.2. Electrophoresis

SDS-PAGE was performed on a 15% gel [10], using Tris–glycine running buffer (25 m M tris, 200 m M glycine, 0.1% SDS, p H 8.3). 30 μg protein was loaded in each well. Protein molecular weight marker (PUREGENE, Genetix) was run simultaneously.

2.4.3. Western blotting

The membrane (Immobilon-PSQ Membrane, PVDF, 0.2 μm from millipore) was cut to appropriate size and dipped in methanol for 1 min, soaked in towbin buffer (25 m M Tris–HCl, 192 m M Glycine, 0.1% w/v, 20% methanol) for 15 min, and sandwiched between blotting paper towels, also soaked in towbin buffer, and placed in the blotting apparatus (semidry, from BIOTECH). Blotting was done at 55 V for 15 min. The membrane was stained with Ponceau to confirm protein transfer and then destained. The gel was stained with Coomassie blue to confirm complete transfer. The blot was then washed with 1× TBST buffer (60.55 M Tris Cl buffer, 29.2 M NaCl, 0.1% Tween 20) and dipped in 5% BSA to block non-specific sites and washed again. It was then dipped in the primary antibody (mouse polyclonal ERAF/AHSP antibody, NOVUS, diluted 1:500) for 15 h, followed by washing with TBST buffer, and then incubation in the HRP conjugated-secondary antibody (goat antimouse IgG h + 1, NOVUS, 1:2000) for 3 h. The blot was again washed and then incubated with the substrate (TMB, Sigma). The image was captured and analyzed using the alpha imager software in the gel documentation system.

3. Results and discussion

Relative quantitation of AHSP in thalassemia major patients, as compared to controls, revealed downregulation in 64% of the patients, upregulation in 16% and was comparable to controls in 20% of the patients (Fig. 1a & b) The expression was not seen to correlate with the age of the patients, or their sex, which supports earlier data [2]. Data suggest that the factors that apparently influence the expression of AHSP are the number of nucleated RBCs, DNA damage in RBC precursors and HbF levels.

3.1. Nucleated RBCs and AHSP level

Beta thalassemia is associated with an increase in alpha-beta globin chain ratio. The free-iron species released from the unpaired alpha chains induces the formation of oxygen radicals that cause cellular damage resulting in hemolysis and ineffective erythropoiesis [11]. This ineffective erythropoiesis causes the release of nucleated RBCs in the circulation [12].

In the present study, nucleated RBCs have been seen in the peripheral blood samples of patients expressing AHSP at par with, or higher than non-thalassemic controls (Fig. 2). The pattern of AHSP expression has been found to be similar to that of hemoglobin, with the highest

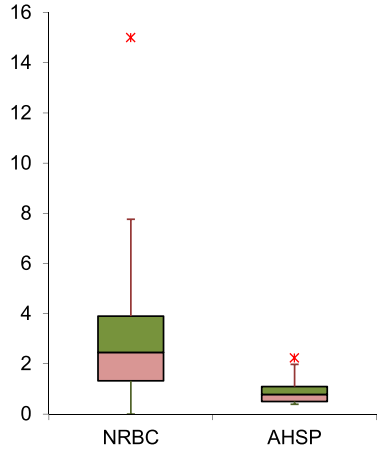

Fig. 2. % nucleated RBCs and fold expression of AHSP in the subjects.