Molecular analysis and association with clinical and laboratory manifestations in children with sickle cell anemia

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ABSTRACT

Objectives: To analyze the frequency of β⁸-globin haplotypes and alpha-thalassemia, and their influence on clinical manifestations and the hematological profile of children with sickle cell anemia.

Method: The frequency of β⁸-globin haplotypes and alpha-thalassemia and any association with clinical and laboratorial manifestations were determined in 117 sickle cell anemia children aged 3–71 months. The confirmation of hemoglobin SS and determination of the haplotypes were achieved by polymerase chain reaction-restriction fragment length polymorphism, and alpha-thalassemia genotyping was by multiplex polymerase chain reaction (single-tube multiplex-polymerase chain reaction).

Results: The genotype distribution of haplotypes was 43 (36.7%) Central African Republic/Benin, 41 (35.0%) Central African Republic/Central African Republic, 20 (17.0%) Rare/atypical, and 13 (11.1%) Benin/Benin. The frequency of the α3.7 deletion was 1.71% as homozygous (−α3.7/−α3.7) and 11.9% as heterozygous (−α3.7/αα). The only significant association in respect to haplotypes was related to the mean corpuscular volume. The presence of alpha-thalassemia was significantly associated to decreases in mean corpuscular volume, mean corpuscular hemoglobin and reticuloocyte count and to an increase in the red blood cell count. There were no significant associations of β⁸-globin haplotypes and alpha-thalassemia with clinical manifestations.

Conclusions: In the study population, the frequency of alpha-thalassemia was similar to published data in Brazil with the Central African Republic haplotype being the most common, followed by the Benin haplotype. β⁸-globin haplotypes and interaction between alpha-thalassemia and sickle cell anemia did not influence fetal hemoglobin concentrations or the number of clinical manifestations.

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Introduction

Sickle cell anemia (Hb SS) is characterized by homozygosity for hemoglobin S (Hb S), consequent to a point mutation in the 6th codon of the \( \beta \)-globin gene on chromosome 11.1,2 It is the most prevalent hereditary hematologic disease in Brazil with elevated morbidity and mortality. It is estimated that 3500 children are born with Hb SS annually, one in every 1000 live births, whereas the frequency of heterozygotes in the general population is 2–8%.3,4 The clinical state of these patients is exceptionally variable, which may be influenced by different factors, such as the haplotypes of the \( \beta^+ \)-globin gene, the presence of alpha-thalassemia, and the fetal hemoglobin level (Hb F).1,2

Haplotypes of the \( \beta^+ \)-globin gene are determined by specific polymorphism patterns in the \( \beta \) gene complex. They originate from different regions of Africa, from which they receive their denominations: CAR or Bantu (Central African Republic), Benin (West Africa), Senegal (West Africa), Cameroon (West Africa) and Saudi-Indian (Asia, India and the Arabian Peninsula). Polymorphism sets not recognized by these classical patterns are referred to as atypical (ATP) and occur at a rate of 5–10%.5,6 In Brazil, due to the forced migration of African descendants, CAR and Benin (BEN) haplotypes are the most common.5,7–11

Alpha-thalassemia is the result of deficient synthesis of \( \alpha \) globin chains in hemoglobin (Hb). The \( \alpha \) chain genes are located on chromosome 16, and normal individuals have two \( \alpha \) genes on each chromosome with this genotype being represented as \( \alpha^+ \alpha^+ \).12 The \( \alpha^3.7 \) deletion, which causes the most common form of alpha-thalassemia in Brazil,13 is the result of an unequal cross-over exhibiting a loss of a 3.7 kb DNA fragment.14 Recent Brazilian studies show different prevalences for the \( \alpha^3.7 \) genotype according to the geographical ancestry of the individuals studied: 4.5% in European and 21.5% in African descendants,15 data similar to those obtained in populations of African descents by Sonati et al.16 Brazilian studies analyzing alpha-thalassemia in individuals with Hb SS present prevalences of \( \alpha^3.7 \) heterozygous individuals of from 17.6 to 28.2%, according to the geographic region.9–11,13,17

The interaction between Hb SS and alpha-thalassemia is associated with an inhibition of Hb S polymerization182,18,19 which decreases hemolytic episodes18,18 entailing reductions in mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) compared to Hb SS individuals without alpha-thalassemia.20 An improvement in the patients’ clinical state is observed with reductions in sickle cell crises.18,21 The presence of alpha-thalassemia is also significantly associated with a reduction in white blood cell (WBC)15 and reticulocyte1,2,17 counts, and increased levels of Hb A2.17,22–25 Although there is no defined relationship, increased levels of Hb F are also reported.5,24

The objective of this study was to assess the prevalence of \( \beta^+ \)-globin haplotypes and alpha-thalassemia in a group of children with Hb SS and their influence on hematological profile and clinical manifestations.

Methods

This was a cross-sectional study with convenience sampling involving 117 of 122 under 6-year-old children with Hb SS, no matter the severity of the disease, attended at the Pediatric Hematology Outpatient Clinic of the Escola Paulista de Medicina, Universidade Federal de São Paulo. The samples were collected from September 2007 to December 2009; the age varied from 3 to 71 months (median age of 35.5 months) and 62 (52.99%) of the children were male. The study was approval by the Research Ethics Committee of the Escola Paulista de Medicina, Universidade Federal de São Paulo.

Exclusion criteria

Patients who had received red blood cell transfusions in the 3 months prior to sample collection and those receiving hydroxyurea were excluded from the study.

Clinical and laboratorial variables

Clinical and laboratorial variables were obtained from the patient’s medical records. The following laboratorial criteria were analyzed: Hb level, hematocrit (Ht), red blood cell (RBC), WBC, MCV, MCH, reticulocyte, and platelet counts, and Hb F levels.

The clinical variables were analyzed considering whether the patients had <3 or ≥3 types of manifestations per year including vaso-occlusive crisis (VOC), acute chest syndrome/pneumonia (ACS/PNM), infections (acute osteomyelitis and urinary tract infection) and episodes of acute splenic sequestration.

Molecular analysis

DNA was extracted from WBCs in peripheral blood using phenol–chloroform extraction and ethanol precipitation.26 The Hb S mutation was confirmed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).27 Single-tube multiplex-PCR was used for alpha-thalassemia genotyping using primers standardized by Chong et al.28 Determination of the haplotypes of the \( \beta^+ \) gene complex was performed by PCR-RFLP analysis using primers as proposed by Sutton et al.29 All tests were conducted using a thermal cycler from Veriti Applied Biosystems® (Foster City, CA, USA).

Statistical analysis

Non-parametric tests were used for statistical analysis taking into account the variability and distribution of the study variables. Kruskal–Wallis with the Dunn test was used to compare more than two samples, and the Mann–Whitney test for two independent samples. Significance was set for alpha errors of 5% (\( p \)-value <0.05); statistically analysis was performed using the InStat-2 (GraphPad®, San Diego, CA, USA) and SigmaStat 3.11 software (Systat® Software, Chicago, IL, USA).
### Results

The frequencies of haplotypes were 57.26% for CAR, 33.33% for BEN, 8.12% for ATP, 0.85% for SAUDI and 0.43% for Senegal. The genotype distribution was CAR/BEN in 43 (36.75%) patients, CAR/CAR in 41 (35.04%), Rare/ATP in 20 (17.09%) and BEN/BEN in 13 (11.11%). In the 20 Rare/ATP patients, the following combinations were identified: BEN/ATP in eight patients (6.84%), CAR/ATP in seven (5.98%), ATP/ATP in two (1.71%), and one individual (0.85%) with the SEN/CAR, SAUDI/CAR, and SAUDI/BEN haplotypes.

Even though the CAR/CAR group presented lower levels of Hb F than the other groups, no statistical difference was observed. MCV was different between the CAR/BEN and BEN/BEN haplotypes (p-value = 0.01) (Table 1). A comparison between different haplotypes showed no difference concerning the number of clinical manifestations presented (p-value = 0.22) (data not shown).

In relation to the occurrence of alpha-thalassemia, two patients (1.71%) were homozygous for the α3.7 deletion and 14 (11.97%) heterozygous, totaling 13.68% of alpha-thalassemia individuals (16/117). The alpha-thalassemia group (homozygotes and heterozygotes) presented significant reductions in MCV, MCH and reticulocyte counts, and had a significant increase in the RBC count compared to the group without the deletion. Although Hb F levels in the alpha-thalassemia group were higher in relation to the group without alpha-thalassemia (18.3%; range: 4.2–31.1 vs. 17.8%; range: 1.8–51.3, respectively) no significance was detected (p-value = 0.84) (Table 2). Regarding the number of clinical manifestations, there was no significant difference between the groups (p-value = 0.66).

Table 3 shows the frequency of alpha-thalassemia in relation to the β5-globin haplotypes. Significant differences were observed in MCV values between individuals with and without alpha-thalassemia in the CAR/CAR and CAR/BEN groups (p-value = 0.002 and 0.017, respectively) (Table 4).

### Discussion

β5-globin gene haplotypes are being studied in Brazil and in other countries with the objective of determining their frequency in different regions and their influence on the clinical manifestations of Hb SS, since this is not yet well characterized. Alpha-thalassemia has also been considered an important modifying factor of clinical severity in Hb SS as it reduces hemolytic episodes and alters the hematological profile of patients.

In patients with Hb SS, the elevated concentration of Hb F protects against vaso-occlusive events, consequently reducing clinical manifestations. Steinberg reported that Hb F is the most powerful disease modifier and Powars’ stated that, in being related to low Hb F levels, the CAR haplotype is associated with more severe clinical manifestations, while SEN and SAUDI haplotypes present high Hb F levels with milder clinical manifestations, and the BEN haplotype presents intermediate Hb F levels and clinical course.
Table 2 – Hematological data expressed as medians (range), according to presence of α3.7 thalassemia in 117 sickle cell anemia children.

|                  | α3.7 thalasemia Absence | α3.7 thalasemia Presence |
|------------------|-------------------------|-------------------------|
|                  | n   | Median (range) | n   | Median (range) |
| Hemoglobin (g/dL) | 98  | 8.5 (6.5–10.5) | 16  | 8.5 (7.1–9.5) |
| Hematocrit (%)   | 98  | 22.0 (14.7–30.7)| 11  | 22.6 (20.1–28.7)|
| RBC (×10^6/L)    | 72  | 2.6 (1.8–4.4)  | 10  | 3.0 (2.5–4.0)  |
| MCV (fL)         | 90  | 83.5 (59.0–95.0)| 12  | 74.4 (62.9–83.7)|
| MCH (pg)         | 89  | 28.8 (22.1–35.2)| 12  | 26.3 (20.0–28.7)|
| WBC (×10^3/L)    | 99  | 18.1 (6.7–32.9)| 12  | 17.2 (7.0–21.8)|
| Platelets (×10^9/L) | 98 | 390 (125–932) | 12  | 396 (285–591) |
| Reticulocytes (%) | 64  | 9.8 (1.5–22.0) | 8   | 4.1 (0.7–13.2) |
| Hemoglobin F (%)  | 77  | 17.8 (1.8–51.3) | 11  | 18.3 (4.2–31.1)|

α3.7 thalassemia: heterozygous and homozygous; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; WBC: white blood cells.

* Mann–Whitney.

In the current study, the CAR haplotype was the most common haplotype encountered, followed by BEN; these data are similar to other studies conducted in the southeast of Brazil. Despite the fact that there was no significant difference in Hb F levels among haplotypes, these values follow the pattern described in the literature, that is, lower values in CAR/CAR and CAR/BEN genotypes, followed by BEN/BEN.

Table 3 – Frequency of α-thalassemia according to β*-globin haplotypes in 117 children with sickle cell anemia.

| Haplotypes   | αα/αα (%) | α3.7 thalassemia (%) |
|--------------|-----------|----------------------|
| CAR/BEN      | 37 (31.62) | 6 (5.13)              |
| CAR/CAR      | 35 (29.91) | 6 (5.13)              |
| BEN/BEN      | 11 (9.4)   | 2 (1.71)              |
| CAR/ATP      | 6 (5.13)   | 1 (0.85)              |
| BEN/ATP      | 8 (6.84)   | 0                     |
| ATP/ATP      | 2 (1.71)   | 0                     |
| SAUDI/CAR    | 1 (0.85)   | 0                     |
| SAUDI/BEN    | 1 (0.85)   | 0                     |
| SEN/CAR      | 0          | 1 (0.85)              |
| Total        | 101 (86.32)| 16 (13.68)            |

α3.7 thalassemia: homozygous and heterozygous; CAR: Central African Republic; BEN: Benin; SEN: Senegal; SAUDI: Asia, India and the Arabian Peninsula; ATP: atypical.

No significant difference was observed in the number of clinical manifestations between the haplotype types, probably due to the fact that the studied population presented a mean age of 37 months, when Hb F levels are still elevated in Hb SS individuals, and many clinical manifestations are not apparent at this age. Another possible factor might be the miscegenation of the Brazilian population and consequent elevated number of individuals with heterozygous haplotypes. A study conducted by Silva Filho et al., with a similar age group, encountered similar results.

The Rare/ATP group consists of different haplotype combinations, mostly involving CAR haplotype heterozygotes (11/25 = 44%) rather than heterozygotes and homozygotes with the ATP haplotype. According to Zago et al., ATP haplotypes can be considered a variation of the Bantu haplotype, and thus we can suppose that they might be related to greater severity of the disease. Although the rare haplotypes (SEN and SAUDI) are associated with a milder clinical course, they did not influence the results obtained as they were identified in only three patients, confirming their low prevalence in the study region.

Some hematological parameters are related to the severity of the disease and are directly implicated in the pathophysiology of Hb SS, hence the importance of assessing their association with haplotypes and alpha-thalassemia. In the comparison between the different haplotype groups, the CAR/CAR group presented significantly lower Hb levels,

Table 4 – Mean corpuscular volume (MCV) presented as median (range) in CAR/CAR and CAR/BEN haplotypes according to the presence of α3.7 thalassemia.

| Haplotypes | α3.7 thalassemia Absence | α3.7 thalassemia Presence |
|           | n   | Median (range) | n   | Median (range) |
| CAR/CAR   | 30  | 85.3 (68.5–95.0)| 6   | 77.4 (59.6–79.6)|
| CAR/BEN   | 32  | 81.4 (59.0–89.2)| 3   | 71.1 (67.2–75.3)|

α3.7 thalassemia: homozygous and heterozygous; CAR: Central African Republic; BEN: Benin.

* Mann–Whitney.
followed by the CAR/BEN. These data are similar to results by Silva Filho et al.\textsuperscript{11} and de Neonato et al.\textsuperscript{24}

Low MCV and MCH values are associated with a reduction in the synthesis of \( \alpha \) chains and related to the effect that alpha-thalassemia exerts on the polymerization of Hb S and on the deformity of sickled cells, making them less dense.\textsuperscript{20} Other than this, there is evidence that microcytosis, per se, exert beneficial effects on vaso-occlusive complications in Hb SS.\textsuperscript{2,21}

The frequency of alpha-thalassemia in the study population was similar to that found in other Brazilian studies performed on patients with Hb SS.\textsuperscript{9,10,17} HB values did not differ between the groups with or without alpha-thalassaemia, similar to the results reported by Silva Filho et al.\textsuperscript{11} and Stevens et al.\textsuperscript{23} Significant reductions in MCV and MCH levels and reticulocyte counts, and a significant increase in the RBC count in \( \alpha 3.7 \) deletion patients confirms data in the literature.\textsuperscript{3,17,22} There was no statistically significant difference in the WBC count between the groups, contrary to other publications that reported a reduction in the WBC count in individuals with alpha-thalassemia.\textsuperscript{17,22}

HB F levels did not show any significant difference between the two groups, similar to results obtained by Silva Filho et al.,\textsuperscript{11} Belisário et al.\textsuperscript{37} and Mouélé et al.,\textsuperscript{32} in studies conducted with similar age groups.

The current study did not observed any influence of alpha-thalassaemia on clinical manifestations, different to other studies where the presence of alpha-thalassaemia preserved splenic function, leading to persistence of splenomegaly in patients with homozygous \( \alpha 3.7 \) deletion.\textsuperscript{31} The fact that HB F is still elevated in the study group might also justify the lack of influence of the deletion in alpha-thalassemia on the clinical manifestations of these patients.

One factor that might be considered as a bias in this study is that data collection of clinical events was from the patients’ medical records, which could be considered deficient. However, the patients were followed-up in the institution, not only in the outpatient clinic but also in the emergency ward and during hospitalization, which reduces or discards bias caused by dependence on the memory of the patient’s family.

Conclusions

The frequency of alpha-thalassaemia in this study was similar to previous studies in Brazil, with the CAR haplotype being the most common, followed by the BEN haplotype. The \( \beta^+ \)-globin gene haplotypes and the presence of alpha-thalassaemia did not influence the levels of HB F or the number of clinical manifestations presented. We believe that a prospective follow-up of these patients will provide more knowledge on the influence of alpha-thalassaemia and the \( \beta^+ \)-globin gene haplotypes on clinical manifestations in Hb SS.

Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1. Powars DR. \( \beta^+ \) gene cluster haplotype in sickle cell anemia: clinical and hematologic features. Hematol Oncol Clin North Am. 1991;5(3):475–93.
2. Steinberg MH. Predicting clinical severity in sickle cell anemia. Br J Haematol. 2005;129(4):465–81.
3. Cançado RD, Jesus JA. A doença falciforme no Brasil. Rev Bras Hematol Hemoter. 2007;29(3):203–6.
4. Fernandes AP, Januário JN, Cangussu CB, de Macedo DL, Viana MB. Mortality of children with sickle cell disease: a population study. J Pediatr (Rio J). 2010;86(4):279–84.
5. Zago MA, Figueiredo MS, Ogo SH. \( \beta^+ \) cluster haplotype predominates among Brazilian blacks. Am J Phys Anthropol. 1992;88(3):295–8.
6. Zago MA, Silva Jr WA, Dalle B, Gualandro S, Hutz MH, Lapoumesoulie C, et al. Atypical \( \beta^+ \) haplotypes are generated by diverse genetic mechanisms. Am J Hematol. 2000;63(2):79–84.
7. Gonçalves MS, Bornfim GC, Maciel E, Cerqueira I, Lyra I, Zanette A, et al. \( \beta^+ \) haplotypes in sickle cell anemia patients from Salvador, Bahia, northeastern Brazil. Braz J Med Biol Res. 2003;36(10):1283–8.
8. Figueiredo MS, Kerbauy J, Gonçalves MS, Arruda VR, Saad ST, Sonati MF, et al. Effect of \( \alpha \) thalassemia and \( \beta \) globin gene cluster haplotypes on the hematological and clinical features of sickle cell anemia in Brazil. Am J Hematol. 1996;53(2):72–6.
9. Lyra IM, Gonçalves MS, Braga JA, Gesteira MF, Carvalho MH, Saad ST, et al. Clinical, hematological and molecular characterization of sickle cell anemia pediatric patients from two different cities in Brazil. Cad Saúde Pública. 2005;21(4):1287–90.
10. Belisário AR, Martins ML, Brito AM, Rodrigues CV, Silva CM, Viana MB. \( \beta-\)Globin gene cluster haplotypes in cohort 221 children with sickle cell anemia or \( S \)-thalassemia and their association with clinical and hematological features. Acta Haematol. 2010;124(3):162–70.
11. Silva Filho IL, Ribeiro GS, Moura PG, Vechi ML, Cavalcante AC, Andrade-Serpa MJ. Manifestações clínicas agudas na primeira e segunda infâncias e características moleculares da doença falciforme em um grupo de crianças do Rio de Janeiro. Rev Bras Hematol Hemoter. 2012;34(3):196–201.
12. Leung WC, Leung KY, Lau ET, Tang MH, Chang V. Alpha-thalassemia. Semin Fetal Neonat Med. 2008;13(4):215–22.
13. Viana MB, Oliveira BM. Alfa-talassemia deve ser considerada no diagnóstico diferencial de anemia na criança. J Pediatr (Rio J). 2011;87(2):180–1.
14. Harteveld CL, Higgs DR. Alpha-thalassaemia. Orphanet J Rare Dis. 2010;5:13–33.
15. Wagner SC, Castro SM, Gonzalez TP, Santin AP, Filippo L, Zaleski CF, et al. Prevalence of common \( \alpha \)-thalassemia determinants in south Brazil: importance for the diagnosis of microcytic anemia. Genet Mol Biol. 2010;33(4):641–5.
16. Sonati MF, Farah SB, Ramalho AS, Costa FF. High prevalence of alpha-thalassemia in a black population of Brazil. Hemoglobin. 1991;15(4):309–11.
17. Belisário AR, Rodrigues CV, Martins ML, Silva CM, Viana MB. Coinheritance of \( \alpha \)-thalassemia decreases the risk of cerebrovascular disease in a cohort of children with sickle cell anemia. Hemoglobin. 2010;34(6):516–29.
18. Tomé-Alves R, Marchi-Salvador DP, Orlando GM, Palharini LA, Imperial RE, Naoum PC, et al. Hemoglobinopathias AS/Alfa talassemia – importância diagnóstica. Rev Bras Hematol Hemoter. 2000;22(3):394–9.
19. Cardoso GL, Guerreiro JF. Molecular characterization of sickle cell anemia in the northern Brazilian state of Pará. Am J Hum Biol. 2010;22(5):573–7.

20. Ballas SK. Effect of α-globin genotype on the pathophysiology of sickle cell disease. Pediatr Pathol Mol Med. 2001;20(2):107–21.

21. Sarnaik SA, Ballas SK. Molecular characteristics of pediatric patients with sickle cell anemia and stroke. Am J Hematol. 2001;67(3):179–82.

22. Mouélé R, Pambouc D, Feingold J, Galácteros AF. Alpha-thalassemia in Bantu population from Congo-Brazzaville: its interaction with sickle cell anemia. Hum Hered. 2000;50(2):118–25.

23. Stevens MC, Maude GH, Beckford M, Grandison Y, Mason K, Taylor B, et al. α-thalassemia and the hematology of homozygous sickle cell disease in childhood. Blood. 1986;67(2):411–4.

24. Neonato MG, Guiloud-Bataille M, Beauvais P, Bégué P, Belloy M, Benkerrou M, et al. Acute clinical events in 299 homozygous sickle cell patients living in France. Eur J Haematol. 2000;65(3):155–64.

25. Adorno EV, Zanette A, Lyra I, Seixas MO, Reis MG, Gonçalves MS. Clinical and molecular characteristics of sickle cell anemia in the northeast of Brazil. Genet Mol Biol. 2008;31(3):621–5.

26. Lee TH, Sakahara NS, Fiebig EW, Huschkorn DF, Johnson DK, Busch MP. Quantification of with cell subpopulations by polymerase chain reaction using frozen whole-blood samples. Viral activation transfusion study. Transfusion (Paris). 1998;38(3):262–70.

27. Steinberg MH, Forget BG, Higgs DR, Nagel RL, editors. Disorders of hemoglobin; genetics, pathophysiology and clinical management. Cambridge, Inglaterra: Cambridge University Press; 2001. p. 953–4.

28. Chong SS, Boehm CD, Higgs DR, Cutting GR. Single-tube multiplex-PCR screen for common deletion determinants of α-thalassemia. Blood. 2000;5(1):360–2.

29. Sutton M, Bouhassia EE, Nagel RL. Polymerase chain reaction amplification applied to the determination of β-like globin gene cluster haplotypes. Am J Hematol. 1989;32(1):66–9.

30. Steinberg MH. Pathophysiologically based drug treatment of sickle cell disease. Trends Pharmacol Sci. 2006;27(4):204–10.

31. Higgs DR, Aldridge BE, Lamb J, Clegg JB, Weatherall DJ, Mayer RJ, et al. Interaction of alpha-thalassemia and homozygous sickle cell disease. N Engl J Med. 1982;306(24):1441–6.