The frequency of $\beta^S$-globin haplotypes in the state of Paraná, Brazil, and clinical manifestations of sickle cell anemia

Frequência dos haplótipos da globina $\beta^S$ no estado do Paraná, Brasil, e manifestações clínicas em pacientes com anemia falciforme

Alexandra M. Watanabe1; Mara A. D. Pianovski2; Luana Lenzi2; Rubens Cat1

1. Centro de Hematologia e Hemoterapia do Paraná (Hemepar), Paraná, Brazil. 2. Universidade Federal do Paraná (UFPR), Paraná, Brazil.

ABSTRACT

Introduction: Haplotypes in the $\beta^S$-globin cluster are named according to their geographical origin as Central African Republic (CAR), Benin (BEN), Senegal (SEN), Cameroon (CAM) and Arab-Indian. They are considered to have influence on the diversity of clinical manifestations in sickle cell anemia (HbSS). Objective: To identify $\beta^S$ haplotypes and genotypes, their frequencies and their probable association with clinical presentation in patients with sickle cell anemia in the state of Paraná. Method: Longitudinal and descriptive study for the definition of haplotypes, and associative study for analysis of their influence on clinical severity. By polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), polymorphic regions of 100 HbSS patients were identified. The association of haplotypes with clinical manifestations was analyzed in a subset of 52 pediatric patients. Results: In the state of Paraná, haplotype frequencies were: CAR: 76%; BEN: 17.5%; SEN: 0.5%; CAM: 0.5% and Atypical (Atp): 5.5%. Genotype frequencies were: CAR/CAR: 62%; CAR/BEN: 20%; CAR/Atp: 6%; CAR/SEN: 1%; CAR/CAM: 1%; BEN/BEN: 6%; BEN/Atp: 3%, Atp/Atp: 1%. The average percentage of fetal hemoglobin (HbF) in CAR/CAR and CAR/BEN patients was higher than in other studies. Clinical manifestations were not influenced by $\beta^S$ haplotypes. Dactylitis and splenic sequestration occurred more frequently in children below 3 years of age. Conclusion: In this study, no association was found between haplotypes and clinical manifestations, probably given the almost absolute predominance of CAR and BEN haplotypes. However, this fact alerts to the possible influence of other polymorphisms and miscegenation in the Brazilian population.

Key words: sickle cell anemia; pediatrics; haplotypes.

INTRODUCTION

Sickle cell anemia is characterized by hemolysis with clinical manifestations resulting from tissue hypoxia and vaso-occlusive phenomena; the severity of clinical manifestations is variable(1). Still in childhood, it is difficult foreseeing the frequency and intensity of these manifestations, what brings uncertainties and insecurity to relatives regarding prognosis. In Brazil, morbidity and mortality have decreased due to early diagnosis reached by neonatal screening programs, to the prophylactic use of antibiotics against invasive bacteria, and to continuous education(2).

The severity of clinical manifestations is attributed to the different concentrations of fetal hemoglobin (HbF) associated with the hemoglobin S (HbS) haplotypes that, historically, originated in African and Asian populations, whose nomenclature was based on geographical location: Benin (BEN), Central African Republic (CAR or Bantu), Cameroon (CAM) and Senegal (SEN), in Africa; and the Arab-Indian type or Asian, in Asia(3-5). The haplotypes can be identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)(6).

Patients with the CAR/CAR genotype present low HbF concentrations and more severe clinical manifestations; those with the BEN/BEN genotype, moderate HbF and clinical manifestations; those homozygous for SEN and Arab-Indian haplotypes are associated with higher HbF concentrations(3, 4, 7) and mild clinical severity.

Considering the hypothesis that haplotypes can influence on the clinical expression of sickle cell disease(3, 4, 8-10), the objective of the present work was to determine the $\beta^S$-globin haplotypes,
The frequency of β-globin haplotypes in the state of Paraná, Brazil, and clinical manifestations of sickle cell anemia

their frequencies and association with clinical manifestations in patients with sickle cell disease in the state of Paraná.

**METHOD**

The study was longitudinal, descriptive and analytical in the definition and frequency of β-globin haplotypes, and associative in the assessment of haplotype influence on clinical severity. Between March 2011 and January 2014, blood samples were consecutively collected from 100 patients with sickle cell anemia (HbSS) in Curitiba, treated at the outpatient clinics of pediatric hematology of Hospital de Clínicas (HC) of Universidade Federal do Paraná (UFPR) (n = 52), and of outpatients clinics of hemoglobinopathy of the coordinating blood center of the state of Paraná — Centro de Hematologia e Hemoterapia do Paraná (Hemepar) (n = 48). Genotyping of β-globin haplotypes was performed at Centro de Genética Molecular e Pesquisa do Câncer em Crianças (Cegem�ac). Clinical and laboratory data were retrospectively collected from records of 52 children treated at HC, born in the state of Paraná, who were not on hydroxyurea during the research. The information was extracted according the consultation protocol for patients with sickle cell anemia in the latest 16 years. Adult patients of Hemepar, who were on hydroxyurea, were just genotyped.

The project was approved by the research ethics committee of HC/UFPR. Participation in the study occurred after signature of the free consent term by the patient and/or legal representative.

The diagnosis of sickle cell anemia was defined through cellulose acetate alkaline electrophoresis, measurement of hemoglobin A2 (HbA2) with elution, and determination of HbF percentage by the alkali-resistance Singer-Chernoff technique. The values considered for analysis were those obtained from patients over 1 year of age, to avoid the influence of physiological variations that happen with this parameter in the first year of life. The assessed clinical manifestations were: cerebrovascular accident (CVA), painful crises, dactylitis, lung diseases, urinary tract infections, and splenic sequestration. The analyzed hematological parameters were: Hb (g/dl), mean corpuscular volume (MCV) (fl), leukocyte count (cells/mm³), platelet count (cells/mm³), reticulocytes (%), and HbF (%), collected from patients at baseline. Blood samples were collected in filter paper and dried at room temperature. From the leukocyte-extracted deoxyribonucleic acid (DNA), polymorphisms in β-globin were identified by the PCR-RFLP technique. Regions of β-globin cluster were amplified: 5'γ', γ', γ', φ, 3 φβ, 5'β. From the polymorphic fragments obtained by the activity of restriction enzymes XmnI, HindIII, HincII and HinfI (Fermentas, Promega) were analyzed according to a pre-established protocol. The undefined haplotypes were analyzed with enzymes HinfI, AvaI and BamHI for polymorphisms in regions ε, β and 3'β, respectively, and considered atypical (Atp).

In order to assess the association of clinical manifestations with age, the patient cohort was divided into two: group 1, made of children aged 0-3 years (n = 52); and group 2, of children older than 3 up to 15,9 years of age (n = 46). Group 2 children are also represented in group 1, but in distinct chronological moments. Among the 52 children, five of group 1 and one of group 2 presented no clinical manifestation until the moment of analysis. The cut-off point at 3 years of age was defined by a quote from the literature about higher frequency of dactylitis in children up to that age.

**Statistical analysis**

Clinical and laboratory data underwent statistical treatment with the program Statistical Package for Social Sciences (SPSS) version 17.0. Descriptive and inferential statistical analyses were carried out, considering the significance level of 5% (p < 0.05). Parametric and non-parametric tests were employed according to data normality.

**RESULTS**

The frequencies of β-globin haplotypes in the state of Paraná were: 76% CAR; 17.5% BEN; 0.5 % SEN; 0.5% CAM and 5.5% Atp. The frequencies of genotypes were: 62% CAR/CAR; 20% CAR/BEN; 6% BEN/BEN and CAR/Atp; 3% BEN/Atp; and 1% CAR/SEN, CAR/CAM and Atp/Atp.

Of the 52 children, 24 (46%) were males, and 28 (54%), females. The median age was 92.5 months (17-191 months). The Figure presents the distribution of children according to age, during the analysis of clinical manifestations.

The verified laboratory parameters (mean + standard deviation) were: Hb = 7.33 ± 0.76 g/dl; MCV = 87.01 ± 6.96 fl; leukocyte count = 15,298 ± 4,088/mm³; platelet count: 400,790 ± 118,386/mm³.

**FIGURE — Distribution of patients with sickle cell anemia according to age**

Ordinate: number of patients in each age, during the analysis; abscissa: age of patients in years.
Table 1 presents the values of these parameters for the different haplotypes.

Dactylitis ($p < 0.001$) and splenic sequestration ($p = 0.001$) were more frequent in the group of children up to 3 years of age, while cardiac alterations, in children over 3 years ($p = 0.05$).

Occurrences of CVA ($p = 0.16$), painful crises ($p = 0.104$) and lung diseases ($p = 0.547$) were not influenced by age (Table 2).

Except for CVA, clinical manifestations were not associated with genotypes (Table 3).

The HbF mean of CAR/CAR genotype was significantly lower than the HbF mean of CAR/BEN genotype (Table 4).
The frequency of β-globin haplotypes in the state of Paraná, Brazil, and clinical manifestations of sickle cell anemia

DISCUSSION

The predominance of CAR (76%) haplotype, followed by BEN (17.5%), in the state of Paraná, is similar to the results found in South and Southeast Regions of Brazil. In Triângulo Mineiro, a region of Minas Gerais, the frequency of CAR haplotype was 61.8% and 22.1% of BEN\(^{(15)}\); in Ribeirão Preto, 66.2% of CAR and 23% of BEN\(^{(10)}\); in the city of São Paulo, 55% of CAR and 34% of BEN\(^{(16)}\); in Rio de Janeiro, 72.9% of CAR and 20.3% of BEN\(^{(17)}\); and in Rio Grande do Sul, 66% of CAR and 27% of BEN\(^{(18)}\).

On the other hand, greater representation of the BEN haplotype is observed in Salvador (55.2%)\(^{(19, 20)}\), in Recôncavo Baiano (52.9%)\(^{(20)}\), and in Ceará (43.2%)\(^{(21)}\). The frequency of HbS haplotypes in the different regions of the country reflects the flow of enslaved Africans in the Brazil of the 18\(^{th}\) and 19\(^{th}\) centuries. Salvador and Rio de Janeiro served as their ports of entry. Those born in the region of Benin, northwest of the African country, arrived in Brazil at the port of Salvador, causing a higher frequency of the BEN haplotype in the North Region of the country. Those born in the present-day Central African Republic arrived in Brazil also through Salvador, but their most frequent entry port was Rio de Janeiro. Later on, they were taken to São Paulo, Paraná, Santa Catarina and Rio Grande do Sul, besides Espírito Santo and north of Rio de Janeiro\(^{(22)}\), justifying the higher frequency of the CAR haplotype in the South and Southeast Regions of the Brazil.

Among the clinical manifestations, it was possible to identify that dactylitis (51%) and splenic sequestration (27.6%) happened most frequently up to 3 years of age (\(p < 0.001\)) (Table 2), as expected\(^{(14, 22)}\). There is a downward bias in identifying manifestations specific of the age group older than 3 years, because some children who at 5 years, for example, still did not present cardiac alterations, can present them in the subsequent years.

No difference was observed on clinical manifestation among haplotypes. One explanation can be the small number of patients with homozygous genotype different from CAR/CAR, with BEN/BEN being just 6%. The CAR haplotype, present in 90% of genotypes, can have promoted the occurrence of more severe clinical manifestations, compatible with CAR. Silva Filho et al. (2012)\(^{(23)}\) also found no clinical association with type of haplotype in the studied population.

The presence of α-thalassemia, which could interfere with prognosis, was not analyzed in this study. However, in the population with sickle cell disease in Paraná, its frequency is just 9.67% [Tormen (2015), data not published], with low probability of altering the observed results.

Higher concentrations of HbF protect patients because they inhibit HbS polymerization, attenuating clinical manifestations\(^{(3, 25-27)}\). In this study, CAR/CAR children (\(n = 32\)) presented lower mean HbF (9.6 ± 6.54) (Table 1) than the patients of group SS (12 ± 7.25) (\(p < 0.001\)), but there was no association of HbF levels with the type of assessed clinical manifestations.

The only identified tendency in this series of patients was the greater occurrence of CVA in CAR/Atp patients (\(p = 0.047\)), data similar to those described by Sarnaik and Ballas\(^{(10)}\). In 41 children, whose first CVA happened in the age group of 5.6 ± 3.2 years, the greatest frequency of the event occurred in those who had at least one Atp allele\(^{(10)}\). Deletions of the α globin chain reduced the occurrence of CVA for they inhibited formation of intracellular polymers; excess of these α chains could be a risk factor for this event\(^{(10)}\). In the state of Rio de Janeiro, children with the CAR/Atp genotype, with mean age of 6.6 years (3.2-15 years), had 15 times more chance [odds ratio (OR) = 15.4] to present CVA than those with other genotypes, but the α globin chains did not influence the event occurrence\(^{(28)}\). The association of CVA with the CAR/Atp haplotype in the state of Paraná must be assessed carefully, given the small size of the sample (\(n = 4\)).

The high average HbF levels in CAR/CAR and CAR/BEN patients are noteworthy, as demonstrated in Table 5, in contrast to initial publications, which characterized these genotypes as more severe for the low HbF levels\(^{(7, 9)}\).

### Table 5 - HbF in HbSS patients from different Brazilian states

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Paraná(^{(20)})</th>
<th>Paraná(^{(28)})</th>
<th>Rio de Janeiro(^{(24)})</th>
<th>Minas Gerais(^{(30)})</th>
<th>Ceará(^{(17)})</th>
<th>Bahia(^{(22)})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 52)</td>
<td>(n = 17)</td>
<td>(n = 79)</td>
<td>(n = 200)</td>
<td>(n = 47)</td>
<td>(n = 77)</td>
</tr>
<tr>
<td>CAR/CAR</td>
<td>9.6 ± 6.54</td>
<td>9.5 (3.8-15.9)</td>
<td>11.9 ± 5.3</td>
<td>17.3 ± 8.4</td>
<td>6.14 ± 5.46</td>
<td>7.544 ± 4.342</td>
</tr>
<tr>
<td>CAR/BEN</td>
<td>16.85 ± 6.28</td>
<td>14.3 (1.2-24.2)</td>
<td>16.1 ± 8.1</td>
<td>19.1 ± 7.7</td>
<td>6.47 ± 3.59</td>
<td>8.146 ± 4.631</td>
</tr>
<tr>
<td>CAR/Atp</td>
<td>16.18 ± 9.89</td>
<td>NR</td>
<td>15.9 ± 14.4</td>
<td>NR</td>
<td>10.88 ± 2.78</td>
<td>4.18 ± 2.25</td>
</tr>
<tr>
<td>BEN/Atp</td>
<td>NR</td>
<td>NR</td>
<td>5.3 ± 0.7</td>
<td>NR</td>
<td>1.43 ± 1.17</td>
<td>NR</td>
</tr>
</tbody>
</table>

**HbF:** fetal hemoglobin; **HbSS:** sickle cell anemia; **α:** present study; **NR:** not reported. **CVA:** cerebrovascular accident; **CAR:** Central African Republic; **BEN:** Benin; **Atp:** Atypical.
In order to explain such discrepancies, the following hypotheses are proposed:

- higher HbF values can be associated with polymorphisms found in the $\beta^+$-car-globin cluster, called atypical CAR$^{(33)}$. Srinivas et al. (1988) identified seven subtypes of CAR haplotypes in patients from the Central African Republic: Bantu A1, Bantu A2, Bantu A3, Bantu A4, Bantu A5, Bantu A6 and Bantu A7$^{(34)}$. These subtypes showed strong correlation between the percentage of $\gamma$ and HbF ($r = 0.093, p < 0.000001$). In that study, the Bantu A4 haplotype with $\gamma$ levels higher than 50% presented HbF of up to 30%$^{(34)}$. Eventually, in Iran, five types of CAR haplotypes were identified: Bantu A1, A2, A2a, A4, A6; associated with the Arab-Indian haplotype, presented high concentrations of HbF ($27.83 \pm 12.32$)$^{(35)}$;

- polymorphisms in the locus control region of HS2 of different haplotypes can modify HbF levels for a certain genotype$^{(2)}$, as verified in CAR/CAR, CAR/BEN and BEN/BEN patients with high HbF$^{(2, 19, 32)}$. The analysis of these polymorphisms and the determination of $\gamma$ concentrations in HbSS patients in the state of Paraná, in the genotypes that presented higher HbF, could be a tool for understanding biomolecular mechanisms;

- polymorphisms in other regions of the $\beta^+$-globin gene also influenced the increase in HbF$^{(28, 36)}$, what was deduced from the observation of carriers of hereditary persistence of fetal hemoglobin$^{(38)}$. Thein et al. (2009) suggested that the quantitative trait loci (QTL) XmnI-HB-G2, linked to the $\beta^+$-globin gene, could be associated with severity of the disease, as well as genes not related to its cluster, such as $HBS1L-MYB$ (6q23) and $BCL11A$ (2p)$^{(36, 37)}$. Recently, association was demonstrated between genes $HBS1L-MYB$ and $BCL11A$ and high levels of HbF in Cameroonian patients with CAM haplotypes$^{(38)}$, confirming the hypothesis that alterations in different genes can influence clinical manifestations in sickle cell anemia$^{(39)}$.

In the initial researches carried out among native Africans, patients homozygous for the SEN haplotype presented mild clinical picture, particularly in relation to hemolysis and high HbF$^{(3)}$. In the present work, the only child with SEN haplotype is heterozygous, presenting the genotype CAR/SEN and the following laboratory results: Hb = 7.57 g/dl, HbF = 12.3%, HbA$_2$ = 2.1%; reticulocytes = 22.1% and MCV = 90.5 fl. Hospitalized at an intensive care unit (ICU), the patient needed exchange transfusions. These results show a severe clinical picture.

In a group of 60 SS Afro-American adult patients, with at least one of the SEN haplotypes, the laboratory tests (mean ± standard deviation) were: Hb (9.6 ± 1.1 g/dl), MCV (91.2 ± 9.2 fl) and HbF (9.9% ± 5.4%)$^{(3)}$. In Rio de Janeiro, a CAR/SEN patient aged 3 years presented HbF = 21.6%; another, a BEN/SEN aged 17 years, HbF = 6.8%$^{(35)}$. In Ceará$^{(21)}$, an adult patient with BEN/SEN genotype presented Hb = 8.9 g/dl, HbF = 13.4% and MCV = 92 fl, values similar to those of the child in the present study. One can observe, therefore, that heterozygosity modifies the expression of the SEN haplotype, traditionally considered to induce mild clinical manifestations.

In the countries where heterozygosity predominates for the different haplotypes, and miscegenation is the rule, the association between haplotype and clinical severity becomes less clear$^{(4, 30)}$. On the other hand, the prognosis of clinical morbidity cannot be given just based on haplotypes$^{(8, 14, 30)}$ and HbF$^{(38)}$. Other genetic factors must be involved, interfering in clinical expression$^{(4, 18, 24, 30)}$ of sickle cell anemia.

Thus, in the patients studied in the state of Paraná, predominance of CAR and BEN haplotypes, the lack of association with the type of clinical manifestations, and the possible influence of miscegenation suggest the need to extend molecular studies, aiming at the comprehension of clinical variability in sickle cell diseases.

**ACKNOWLEDGEMENTS**

We thank Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Paraná for the sponsorship.
RESUMO

Introdução: A variabilidade nas manifestações clínicas da anemia falciforme (HbSS) pode ser influenciada pelos haplotípos no grupamento da globina β, nomeados de acordo com a origem geográfica: República Centro-Africana (CAR), Benin (BEN), Senegal (SEN) Camarões (CAM) e Árabe-indiano. Objetivo: Identificar haplotípos e genótipos da globina β, suas frequências e as possíveis associações com manifestações clínicas em pacientes com anemia falciforme no estado do Paraná. Método: Estudo longitudinal e descritivo na distribuição dos haplotípos e associativo na análise da influência destes sobre as manifestações clínicas. Identificaram-se as regiões polimórficas da globina β de 100 pacientes HbSS pela técnica da polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). A associação dos haplotípos com as manifestações clínicas foi analisada em um subgrupo de 52 pacientes pediátricos. Resultados: As frequências dos haplotípos foram CAR: 76%; BEN: 17,5%; SEN: 0,5%; CAM: 0,5% e Atípico (Atp): 5,5%. Os genótipos foram CAR/CAR: 62%; CAR/BEN: 20%; CAR/Atp: 6%; CAR/SEN: 1%; CAR/CAM: 1%; BEN/BEN: 6%; BEN/Atp: 3% e Atp/Atp: 1%. A porcentagem média de hemoglobina fetal (HbF) dos pacientes CAR/CAR e CAR/BEN foi maior que em outros estudos. Os haplotípos da globina β não tiveram influência nas manifestações clínicas. A dactilite e o sequestro esplênico ocorreram com mais frequência nas crianças abaixo de 3 anos de idade. Conclusão: Na população estudada, não foi possível identificar associação dos haplotípos com as manifestações clínicas. Esse fato pode ser decorrente do predomínio quase absoluto dos haplotípos CAR e BEN, de diferentes polimorfismos e da miscigenação da população brasileira.

Unitermos: anemia falciforme; pediatria; haplotípos.

REFERENCES


CORRESPONDING AUTHOR
Alexandra Mitiru Watanabe
Travessa João Prosdocimo, 145; Alto da XV; CEP: 80045-145; Curitiba-PR, Brasil; e-mail: alemwatanabe@hotmail.com.