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Haplotypes βs-globin and its clinical-haematological correlation in patients with sickle-cell anemia in Triângulo Mineiro, Minas Gerais, Brazil

Haplótipos da βs-globina e sua correlação clínica-hematológica em portadores de anemia falciforme no Triângulo Mineiro, Minas Gerais, Brasil

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ABSTRACT

Introduction: Sickle-cell anemia (SCA) is the most severe form of sickle-cell disease, and is characterized by homozygous hemoglobin S (α2βS2). **Objective:** Determine the haplotypes frequency in patients with SCA and their correlation with clinical and hematological profile. **Method:** We performed a retrospective descriptive study by reading the charts and a cross-sectional study for molecular analysis to determine the haplotypes of the gene pS globin in 61 patients with sickle-cell anemia (SS) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), using restriction endonucleases Xmn I, Hind III, Hinf I and Hinc II for analysis of six polymorphic sites in the beta cluster. **Result:** The genotypes were Central African Republic (CAR)/CAR (50.8%), CAR/Benin type (BEN) (13.1%), CAR/Cameroon type (CAM) (1.6%), CAR/atypical (ATP) (13.1%), BEN/BEN (13.1%), BEN/ATP (4.9%) and ATP/ATP (3.3%). Among the analyzed chromosomes, 64.8% were CAR type, 22.1% were BEN, 12.3% ATP and 0.8% CAM. Levels of fetal hemoglobin (HbF) were significantly lower in CAR/CAR than in ATP/ATP, BEN/ATP and CAR/BEN. No association was observed between the different genotypes and clinical manifestations. **Conclusion:** Despite the lack of association between genotypes and clinical profiles, higher frequency of clinical events was observed in patients with at least one type of CAR chromosome. A significant association was also observed between lower average levels of HbF and CAR/CAR genotype compared to other genotypes.

Key words: sickle-cell anemia; haplotypes; fetal hemoglobin.

INTRODUCTION

Sickle-cell anemia (SCA) is caused by homozygous hemoglobin S ($\alpha 2\beta^S 2$) that derives from a point mutation in the β-globin (β^S) chain gene, located on chromosome 11, which leads to the replacement of adenine (A) for thymine (T) at codon 6 (GAG → GTG), resulting in the switch from glutamic acid amino acid to valine in the sixth position of the β^S[1].

It is clinically characterized by chronic hemolytic anemia and repeated vaso-occlusive episodes[2]. As it is extremely variable, the severity of clinical and hematological manifestations may range from a serious illness to an almost asymptomatic condition only accidentally detected. Its hematological features, as well as clinical severity, are affected by variations in the levels of fetal hemoglobin, simultaneous presence of α-thalassemia, deficiency of the glucose-6-phosphate dehydrogenase enzyme, haplotypes related to the β-globin gene cluster, and endothelial dysfunction[3-5].

The set of polymorphic regions of a chromosome is referred to as haplotype[6]. Haplotypes have been useful markers for anthropological studies and to define the flow of βS allele in human populations. They have different ethnic and geographical origins: the Central African Republic (CAR), or Bantu kind, in South-Central and Eastern Africa; the Benin type (BEN), originated in the African Midwest; the Senegal type (SEN), characteristic of Atlantic Africa; the Cameroon type (CAM), found within the geographical boundaries of that country and at a small part of the west coast of Africa; and the Arabian-Indian or Asian, present in the Arabian Peninsula and India. Those that do not correspond

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to the five standard types commonly associated with βS gene are called atypical[7].

The different haplotypes of SCA are related to variable levels of fetal hemoglobin (HbF) and, consequently, also to varied clinical features: the SEN and Arab-Indian haplotypes are associated with high levels of HbF (> 15%) and a milder course of disease; BEN and CAM, to median HbF levels (5% to 15%) and an intermediate clinical course; the Bantu or CAR shows decreased levels of HbF (< 5%) and more severe clinical signs[8].

The incidence of SCA and the absence of a study on the frequency of haplotypes in our region justify the conduction of this work, aiming to determine the haplotypes of patients with SCA treated at the Hematology Service of the university hospital, and to establish a possible association of these haplotypes with clinical and hematologic manifestations.

METHODS

The target population of this study consisted of SCA patients treated at the clinical hospital of Universidade Federal do Triângulo Mineiro (UFTM) and at Hemocentro Regional de Uberaba, state of Minas Gerais, in the period 2008-2012. This is a quantitative cross-sectional analytical study of institutional base, carried out in two steps.

The first step was performed retrospectively. The records of the research participants in the studied period were read considering variables such as age, gender, origin, use of hydroxyurea (HU), diagnosis by neonatal screening, occurrence of major clinical events (vaso-occlusive crisis, leg ulcers, stroke, gallstones, acute chest syndrome, splenic sequestration, acute insufficiency, history of transfusion and hospitalization), and hematological data (erythrocytes, hemoglobin, hematocrit, platelets and HbF), provided that the patients lie off the period of painful crisis.

The second stage of the study was performed transversely. From each of the 61 patients with SCA, homozygous SS, 10 ml of peripheral blood were collected by venipuncture into sterile collection vacuum tubes containing ethylenediaminetetraacetic acid (EDTA), to determine haplotype of βS globin gene. DNA extraction was performed by the phenol-chloroform method. For the identification of haplotypes βS mutation, we used the technique of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and analyzed six polymorphic restriction sites following the method of Sutton *et al*. (1989)[9]: 1) 5'γG XmnI; 2) HindIII γ^{G}; 3) Hind III γ^{A}; 4) Hinc IIψβ; 5) Hinc II 3'δ; and 6) Hinf I 5'β.

For statistical analysis, the categorical variables were examined by a descriptive analysis based on absolute and percentage frequencies; and the numeric variables, by the descriptive measures based on centrality and dispersion. Hematologic parameters were compared between groups of genotypes (CAR/CAR; CAR/BEN; CAR/atypical (ATP); BEN/BEN; BEN/ATP and ATP/ATP), using the analysis of variance (ANOVA). The association of clinical manifestations with groups of haplotypes was checked by the chi-square test. The level of statistical significance for all tests was 5% ($p < 0.05$). The normality of data was checked by the Kolmogorov-Smirnov test; and homogeneity of variances, by the Bartlett test.

The research project was approved by the ethics committee of UFTM, as protocol 1949/2011; and by Fundação Hemominas, under registration number 324/2011. All study subjects signed a free informed consent term.

RESULTS

The epidemiological profiles of the patients participating in the study are shown in **Table 1**.

TABLE 1 – Patients' characteristics in the study

Variables	*n* (%)
Age	
3-15 years	19 (31.1)
16-30 years	24 (39.3)
31-50 years	15 (24.6)
Over 50 years	3 (4.9)
Gender	
Female	41 (67.3)
Male	20 (32.7)
Time since diagnosis	
Up to 6 months	24 (39.3)
7 months to 2 years	13 (21.3)
3-10 years	11 (18)
11-18 years	4 (6.5)
Over 50 years	1 (1.6)
Records without date of diagnosis	8 (13.1)
Guthrie test	
Performed	19 (31.1)
Not performed or no record information	42 (68.9)
Origin	
Uberaba (MG)	26 (42.6)
Mesoregion of Triângulo Mineiro and Alto Paranaíba (MG)	21 (34.4)
North of Minas Gerais (MG)	5 (8.2)
São Paulo state (SP)	5 (8.2)
Goiás state (GO)	2 (3.2)
Bahia state (BA)	1 (1.6)
Rondônia state (RO)	1 (1.6)

The occurrence of the haplotypes and combinations thereof are shown in **Table 2**.

Twenty-seven patients (44.3%) had a history of HU use sometime in the studied period; however, attendance and adherence to treatment were neither monitored nor evaluated. Among these patients, 13 (48.1%) were identified as clearly predominant CAR/CAR genotype carriers. In the multiple comparison of the hematologic profile with seven groups of genotypes, only HbF showed a statistically significant difference (**Table 3** and **Figure**).

When HbF levels between the different genotypes were compared, we observed that CAR/CAR presented the lowest value and was statistically significant when compared with ATP/ATP ($p = 0.03$), BEN/ATP ($p = 0.0006$) and CAR/BEN ($p = 0.0001$). We also emphasize that BEN/ATP had the highest concentration of mean HbF levels among the genotypes and was statistically significant when compared with BEN/BEN ($p = 0.007$), CAR/ATP ($p = 0.004$) and CAR/CAR ($p = 0.0006$).

The associations between genotypes and the presence of clinical complications showed no statistically significant difference, but individuals with the homozygous CAR haplotype showed higher frequency of clinical events. Consolidating these findings, we also found that the group of patients with a CAR chromosome had also increased frequency and severity of clinical events in relation to the groups of patients BEN/BEN, BEN/ATP and ATP/ATP, as shown in **Table 4**.

TABLE 2 – Distribution of genotypes and haplotypes

Genotypes	*n* (%)	CAR	BEN	CAM	ATP
ATP/ATP	2 (3.3)	-	-	-	4
BEN/ATP	3 (4.9)	-	3	-	3
BEN/BEN	8 (13.1)	-	16	-	-
CAR/ATP	8 (13.1)	8	-	-	8
CAR/BEN	8 (13.1)	8	8	-	-
CAR/CAR	31 (50.8)	62	-	-	-
CAR/CAM	1 (1.6)	1	-	1	-
Total	61 individuals	79 (64.8)	27 (22.1)	1 (0.8)	15 (12.3)

Haplotypes – chromosomes 122 (100%).
CAR: Central African Republic; BEN: Benin; CAM: Cameroon; ATP: atypical.

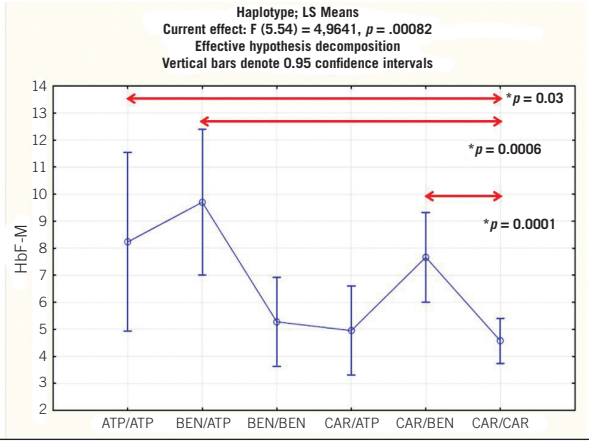

FIGURE – *Representation of average HbF level in genotypes*

F: fetal; HbF-M: mean fetal hemoglobin; ATP: atypical; BEN: Benin; CAR: Central African Republic; $p < 0.05$.

TABLE 3 – Hematologic characteristics in association with genotype

Genotypes	Erythrocytes (× 10^9/l)	Hemoglobin (g/dl)	Hematocrit (%)	Platelets (× 10^9/l)	HbF (%)
CAR/CAR	2.6 ± 0.1	8.1 ± 1.1	23.3 ± 3.7	439,878 ± 156,378	4.6 ± 0.4
CAR/BEN	2.8 ± 0.2	8.2 ± 1.1	23.3 ± 2.9	336,315 ± 120,972	7.7 ± 0.8
CAR/CAM	3.4 ± 0.5	7.3 ± 1	21.2	160,966	7.2
CAR/ATP	2.5 ± 0.2	8.1 ± 0.8	23.6 ± 2.6	446,694 ± 242,737	4.9 ± 0.8
BEN/BEN	2.4 ± 0.5	8.2 ± 1.1	23.3 ± 3	347,895 ± 92,743	5.3 ± 0.8
BEN/ATP	2.7 ± 0.5	8.8 ± 1.1	20.1 ± 2.5	300,111 ± 89,881	9.7 ± 1.3
ATP/ATP	2.9 ± 0.36	8.9	26.6	238,600	8.2 ± 1.6
p value (ANOVA)	0.44	0.77	0.64	0.98	0.008*

The results were reported in average values ± standard deviation. ANOVA test was used for multiple comparison of means between haplotypes, $*p < 0.05$.
HbF: fetal hemoglobin; CAR: Central African Republic; BEN: Benin; CAM: Cameron; ATP: atypical; ANOVA: analysis of variance.

TABLE 4 – Clinical manifestations in relation to genotype (n = 61 patients)

Clinical manifestations	CAR/CAR (*n* = 31)	CAR/BEN (*n* = 13)	CAR/CAM (*n* = 1)	CAR/ATP (*n* = 8)	BEN/BEN (*n* = 8)	BEN/ATP (*n* = 3)	ATP/ATP (*n* = 2)	Total
Gallstones ($p = 0.79$)	13 (46.4)	3 (10.7)	-	4 (14.3)	5 (17.8)	2 (7.1)	1 (3.6)	28 (100)
Leg ulcers ($p = 0.32$)	5 (45.4)	1 (9.1)	-	1 (9.1)	1 (9.1)	2 (18.2)	1 (9.1)	11 (100)
Acute chest syndrome ($p = 0.3$)	10 (66.7)	1 (6.7)	1 (6.7)	1 (6.7)	1 (6.7)	1 (6.7)	-	15 (100)
Stroke ($p = 0.76$)	5 (62.5)	-	-	1 (12.5)	2 (25)	-	-	8 (100)
HF ($p = 0.83$)	8 (47)	3 (17.6)	-	3 (17.6)	2 (11.7)	1 (5.9)	-	17 (100)
HT ($p = 0.36$)	26 (51)	5 (9.8)	1 (2)	7 (13.7)	8 (15.7)	2 (3.9)	2 (3.9)	51 (100)
HH ($p = 0.43$)	25 (49)	6 (11.8)	1 (2)	6 (11.8)	8 (15.7)	3 (5.9)	2 (3.9)	51 (100)

Analysis using chi-square test, $p < 0.05$. *Values presented as n (%).*
CAR: Central African Republic; BEN: Benin; CAM: Cameron; ATP: atypical; HF: heart failure; HT: history of transfusion; HH: history hospitalization.

DISCUSSION

The predominant age group in this study was 3-30 years, corresponding to 70.4% of patients. Only three (4.9%) were older than 50 years. These findings are similar to those described in the literature, which report a shortened life expectancy in this population[10]. A study by Martins *et al*. (2010)[11], in our region, revealed predominant age of 0-29 years (82.5%) and a small contingent over 40 years (8.7%), suggesting that sickle-cell patients die early in Brazil.

We had a patient diagnosed with SCA later, after 52 years old, what characterizes the phenotypic heterogeneity of this disease[4].

As to identification of haplotypes in relation to chromosomes, there was a clear predominance of the CAR haplotype (64.8%) compared to BEN (22.1%), ATP (12.3%) and CAM (0.8%). Findings are similar to those described in different regions of Brazil. A study conducted in Ribeirão Preto (SP) revealed a predominance of the CAR haplotype (66.2%) compared to BEN (23%) in individuals with SCA[12]. The predominance of CAR, followed by BEN, was also observed in two other studies, revealing frequencies of 62.2% and 73.1% for the CAR versus 23% and 25.4% for BEN, respectively[13,14]. In Rio de Janeiro, CAR (54%) was more frequent than BEN (44.6%)[15], as observed in Porto Alegre[16], where the identified frequency was 79.6% for CAR and 18.4% for BEN. In Pernambuco, the CAR frequency was 79.1%[17]; in Rio Grande do Norte, 75.5%[18]; and in Fortaleza, 66.2%[19]. Nevertheless in Bahia, in the Northeast Region, we found the prevalence of BEN (48.8%)[20].

The CAM haplotype in this study showed a low rate (0.8%), similar to those frequencies described in Salvador (BA), Belém (PA), and Pernambuco (PE): 1.2%, 1.3% and 0.8%, respectively[17,21,22].

The presence of 12.3% of atypical chromosomes was similar to the 11.8% occurrence observed by Silva *et al*. (2009)[19]. These findings probably reflect the various genetic mechanisms of association with the sickle gene, i.e. the atypical differ from the five common haplotypes observed in the world, confirming the hypothesis that these different structures are generated by recombination, specific replacement and/or non-reciprocal transfers between common preexisting haplotypes instead of new mutations in the β-globin genes[23]. We emphasize that a patient diagnosed at age 52 in our study showed the ATP/ATP genotype, suggesting, once again, the heterogeneity of the disease.

The highest frequency of the BEN haplotype documented in Bahia[20], different from that found in other regions of Brazil, was similar to those in other countries, such as Venezuela (51.1%) and Cuba (51.0%)[3]. In Jamaica and the United States there are reports of the apparent predominance of this haplotype, with frequencies of 72% and 62%, respectively[12].

Regarding the combinations of haplotypes, the CAR/CAR genotype predominated (50.8%), followed by CAR/ATP, CAR/BEN and BEN/BEN with the same frequencies (13.1%). These findings are consistent with those by Cabral *et al*. (2010)[18], 58.5% for CAR/CAR and 16.9% for CAR/BEN. By contrast, our results were not similar to those described by Belisário *et al*. (2010)[24], in which 39.4% were CAR/CAR, 33.2% CAR/BEN, 23.6% BEN/BEN, 0.9% CAR/ATP, 0.9% BEN/ATP and 0.9% BEN/Arab-Indian. This difference might be explained by the size of the state of Minas Gerais and its territorial boundary with several other states, enabling considerations of interest on the origin and internal migration of Africans in Brazil and in our state.

Nine of the 61 patients (14.7%) came from other states: five from São Paulo (two CAR/CAR, two CAR/BEN and one ATP/ATP), two from Goiás (CAR/CAR, CAR/ATP), one from Rondônia (BEN/BEN) and one from Bahia (BEN/ATP). We believe that the frequency of these genotypes did not impact on our results, because the CAR haplotype also showed a higher frequency in this subgroup. Just as expected, in the state of Bahia, where there is a higher incidence of the BEN haplotype, one patient had the combination BEN/ATP.

Regarding the hematological profile, just HbF showed average values with significant differences among the seven groups of genotypes. The finding of the significant decrease in the average levels of HbF in the CAR/CAR genotype relative to other genotypes of our study is similar to those described in the literature, which are also associated with greater clinical severity[25]. In a study by Fleury[15], as shown in our results, there was significant difference only for HbF among different genotypes.

The heritage of at least one CAR chromosome is associated with more serious pictures than those related to the presence of other haplotypes. Therefore, the genotypes that have the CAR haplotype in common are assumed to be related to similar laboratory and clinical courses[26]. In our study, we observed a higher frequency of clinical complications in patients with at least one CAR chromosome, what is consistent with Cançado[27], who reported a twofold increase in the relative risk of stroke and a fivefold risk for kidney complication when compared to CAR-negative patients.

Thus, the CAR haplotype has the worst prognosis, with greater severity of clinical complications, more hospitalizations and increased morbidity and mortality[28]. Although we found no significant association between the CAR/CAR genotype and clinical events, findings similar to those described by Silva Filho *et al*. (2012)[29], we observed that 48.1% of patients with this genotype had a history of

using HU, what may be indicative of clinical severity. The absence of this association can be attributed to a small sample, heterogeneity of age, the small group of patients with certain genotypes, and the quality of clinical information, which is controlled, because this is a retrospective study of medical records.

CONCLUSION

In the present study, as in most national reports, the CAR haplotype was predominant, followed by BEN. On associations between clinical/hematological profile and the different genotypes, the only significant finding was a decrease in the average level of HbF in the CAR/CAR genotype compared with other genotypes. However, higher frequency of clinical events in patients having one or two CAR chromosomes was observed. This was the first work in the Triângulo Mineiro region, and it revealed a small variation in the frequency of haplotypes in relation to that found in Belo Horizonte. It may contribute to determining the flow of βS-globin in the state of Minas Gerais. In addition, this study can serve as a possible tool for anthropological studies of African origin in our state and country.

RESUMO

Introdução: *A anemia falciforme (AF) é a forma mais grave da doença falciforme, sendo caracterizada por homozigotos de hemoglobina S (α2βS2).* ***Objetivo:*** *Determinar a frequência dos haplótipos de pacientes com AF e sua correlação com o perfil clínico e hematológico.* ***Método:*** *Realizado estudo descritivo e retrospectivo, por meio da leitura dos prontuários, e estudo transversal para análise molecular a fim de determinar os haplótipos da globina do gene pS de 61 pacientes com AF (SS) por reação em cadeia da polimerase-polimorfismo de fragmentos de restrição (PCR-RFLP), utilizando endonucleases de restrição Xmn I, Hind III, Hinf I e Hinc II para análise de seis locais polimórficos no* cluster *beta.* ***Resultado:*** *Os genótipos foram Central African Republic (CAR)/CAR (50,8%), CAR/Benin* type *(BEN) (13,1%), CAR/Cameroon* type *(CAM) (1,6%), CAR/*atypical *(ATP) (13,1%), BEN/BEN (13,1%), BEN/ATP (4,9%) e ATP/ATP (3,3%). Dos cromossomos analisados, 64,8% eram do tipo CAR; 22,1%, BEN; 12,3%, ATP; e 0,8%, CAM. Os níveis de hemoglobina fetal (HbF) foram significativamente menores no CAR/CAR em relação a ATP/ATP, BEN/ATP e CAR/BEN. Não houve associação entre os diferentes genótipos e as manifestações clínicas.* ***Conclusão:*** *Apesar da falta de associação entre genótipos e perfis clínicos, foi observada maior frequência de eventos clínicos em pacientes com pelo menos um tipo de cromossomo CAR. Observou-se também associação significativa dos níveis médios mais baixos de HbF em genótipo CAR/CAR, em comparação com outros genótipos.*

Unitermos: *anemia falciforme; haplótipos; hemoglobina fetal.*

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