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Analysis of *BCL11A* gene polymorphisms and hemolysis parameters in patients with sickle-cell disease

Análise dos polimorfismos do gene BCL11A *e parâmetros de hemólise em pacientes com anemia falciforme*

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ABSTRACT

Introduction: Patients with sickle-cell disease (SCD) present chronic hemolysis with increased serum biomarkers. Genetic polymorphisms of the *BLC11A* gene modulate fetal hemoglobin (HbF), thus reducing hemolysis. **Objective:** To associate the polymorphisms of *BCL11A* gene with the hemolysis markers: reticulocyte, bilirubin, uric acid, lactate dehydrogenase (LDH), and methemoglobin (MetHb) in SCD patients. **Methods:** The study included 45 patients with SCD of both sexes using hydroxyurea (HU), treated at a Hospital in Fortaleza, Ceará, Brazil, along with 80 healthy individuals as the control group. MetHb, uric acid, and bilirubin measurements were carried out with the spectrophotometric method, and LDH with a kinetic method, a reticulocyte count by a manual method; and evaluation of *BCL11A* polymorphisms, in real time polymerase chain reaction (PCR). Data were analyzed using the statistical software GraphPad Prism. The level of significance was set at $< 5\%$. **Results:** In region rs7557939 of the *BCL11A* gene genotype, A/G showed a significant increase of MetHb ($p = 0.0297$), and the A/A genotype showed high concentration of LDH ($p = 0.0316$) in the same region. The use of HU at doses ≥ 10 mg/kg/day showed a decrease of LDH ($p = 0.02$), and treatment for > 50 months was linked to the reticulocyte count ($p = 0.0155$). **Conclusion:** Polymorphisms in the rs7557939 region of the *BCL11A* gene appear to somehow interfere in the clinical setting of patients with SCD, suggesting relation with the concentration of MetHb and LDH. This study pioneered an investigation into the association of hemolysis biomarkers with *BCL11A* gene polymorphisms in SCD.

Key words: sickle-cell anemia; hemolysis; biological markers; genetic polymorphism; fetal hemoglobin.

INTRODUCTION

Sickle-cell disease (SCD) is a genetic blood disease characterized by point mutation in the β-globin gene. The "sickle" form of erythrocytes lead to vaso-occlusion and hemolysis, its main clinical manifestations[1-3]. Chronic hemolysis causes vascular imbalance, with a decreased half-life of red blood cells, reflecting an increase in hemolysis biomarkers; these are used in clinical practice as prognostic markers of SCD and careful monitoring of treatment[4, 5].

Studies have shown an increase in the reticulocyte count in patients with SCD, as this parameter is associated with an increased hemolytic process. Methemoglobin (MetHb) is released during the process, since its concentration is elevated in SCD patients, as shown in several studies[6-8]. Lactate dehydrogenase (LDH) is used as a hemolysis marker and is usually high in patients with SCD, i.e., at a steady state. Increased concentration of LDH is associated with an increased incidence of priapism and leg ulcers in SCD[6, 7, 9, 10]. In SCD, hyperuricemia may occur due to impaired renal tubular dysfunction or the reuse of nucleic acids in hemolysis[6, 7, 11]. SCD patients experience a chronic hemolytic process due to the premature death of red blood cells. Jaundice is often observed in these patients, reflecting the presence of hyperbilirubinemia, especially at the expense of indirect bilirubin (unconjugated)[12].

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Hydroxyurea (HU) is the drug of choice for SCD treatment. In addition to promoting an increase of concentrated hemoglobin F (HbF) by inhibiting hemoglobin S (HbS) polymerization, it reduces hemolysis, expression of adhesion molecules, anti-inflammatory and non-aggregating actions, contributing to the reduction of an occlusive crisis, and frequency of mortality and hospitalization. HU use in patients may exhibit variability in therapeutic response, due to modulators of the HbF concentration, haplotype groups of β-globin, co-association of alpha-thalassemia and presence of polymorphisms such as *XmnI*, and *BCL11A HBS1L-MYB 3*. Furthermore, the response variability can be further associated with variation in drug metabolism[13, 14].

Genetic studies show the presence of single nucleotide polymorphisms (SNPs), which are responsible for 20% to 50% of changes in HbF levels in patients with SCD or thalassemia, and in β and normal individuals. These polymorphisms occur in the *BCL11A* gene, located on a chromosome, which encodes a transcription factor modulating the synthesis of HbF (γ-globin gene expression). Several studies show the relationship between HbF concentration and polymorphisms on the *BCL11A* gene. According to various studies, the regions of the *BCL11A* gene are extremely important in modulating the expression of HbF, such as rs11186868, rs7557939, and rs4671393; however, there is still no consensus on the specific region or polymorphism that would be related to differences in the concentration of HbF[13, 15].

Diversified works have been reported on the association between the *BCL11A* gene and HbF concentration; there are no studies about an influence of the gene on hemolytic parameters. We tried to verify an association between polymorphisms of the *BCL11A* gene and hemolysis (biomarkers like reticulocytes, bilirubin, uric acid, LDH, and MetHb in SCD and HU patients).

METHODS

Patients

This is a cross-sectional, analytical study with 45 SCD adult outpatients at the University Hospital Walter Cantídio, in Fortaleza, Ceará, Brazil. All patients signed an informed consent, according to protocol (no. 706.154) approved by the Ethics Committee of Universidade Federal do Ceará (UFC). The research was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Eligibility criteria included volunteers with SCD molecular diagnosis (HbSS), age ≥ 18 years, in treatment with HU at baseline, according to Ballas criteria[16]. The control group had 80 blood donors (HbAA) who were healthy, without any clinical comorbidities.

Analysis of hemolysis biomarkers

The MetHb level was measured by a spectrophotometric method, like that proposed by Naoum, Radispiel, and Moraes[17]. The measurements of LDH, uric acid, and bilirubin (total and direct) were made with specific kits such as Labtest®, following methods suggested by the manufacturer. The indirect bilirubin levels were determined by subtraction of total bilirubin from direct bilirubin values. The reticulocyte count was done manually, with supravital staining[18].

Molecular biological analysis

Deoxyribonucleic acid (DNA) was isolated from leukocytes collected in a tube with anticoagulant ethylenediaminetetraacetic acid (EDTA), using a specific kit called Axygen® (Union City, CA, USA). The identification of polymorphisms on the *BCL11A* gene was performed by molecular biology with real-time polymerase chain reaction (PCR) and TaqMan® (Life Technologies, Carlsbad, CA, USA), which has used DNA samples and primer specific for the gene. The analysis of polymorphisms in the *BCL11A* gene was performed through the detection of these gene regions: rs4671393, rs7557939, and rs11886868 (**Table 1**). The amplification process was performed with prior activation of the enzyme at 95°C for 10 minutes; this included 40 cycles of DNA denaturation at 92°C for 15 seconds, while annealing an extension (pairing of the oligonucleotide) at 60°C for one minute.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0. We first used the D'Agostino-Pearson test to verify normality of the data. We used statistical tests like Mann-Whitney (for nonparametric variables) and an unpaired *t*-test (for parametric variables) to compare hemolysis biomarkers in SCD patients and a control group (comparing biomarker concentration according to dose and use of HU). To compare biomarkers between the *BCL11A* genotypes, the analysis of variance (Anova) and the Tukey post-hoc test were used as well.

TABLE 1 – Polymorphisms and regions of the *BCL11A* gene

Region	Wild type	Mutant
rs4671393	A	G
rs7557939	G	A
rs11886868	C	T

RESULTS

There was a significant increase in reticulocyte count, MetHb, LDH, and bilirubin levels in SCD patients compared to the control group ($p < 0.0001$) (**Table 2**).

TABLE 2 – Concentration of reticulocytes, MetHb, uric acid, LDH, and bilirubin (BT, BD, and BI) in patients with SCD and the control group

	SCD ($n = 45$)	Control ($n = 80$)	p-value
Reticulocyte (%)	6.637 ± 3.511	0.8224 ± 0.574	< 0.0001*
MetHb (%)	4.009 ± 1.272	2.682 ± 0.6041	< 0.0001*
Uric acid (mg/dl)	4.036 ± 1.712	3.832 ± 1.026	0.555
LDH (U/l)	670.5 ± 309.7	312.6 ± 87.47	< 0.0001*
DB (mg/dl)	0.5413 ± 0.324	0.1439 ± 0.057	< 0.0001*
IB (mg/dl)	1.29 ± 1.123	0.3216 ± 0.156	< 0.0001*a
TB (mg/dl)	1.76 ± 1.251	0.4655 ± 0.2062	< 0.0001*

**The p-value was obtained by the unpaired t-test and the Mann-Whitney test; a results presented as mean ± standard deviation, which is significant at the 0.05 level. MetHb: methemoglobin; LDH: lactate dehydrogenase; DB: direct bilirubin; IB: indirect bilirubin; TB: total bilirubin.*

In the rs7557939 region of the *BCL11A* gene, three genotypes were studied: A/A, A/G, and G/G. Genotype association with hemolytic biomarkers showed an increased MetHb production in genotype A/G, compared to that of A/A ($p = 0.0297$). In the same region, an increase in LDH concentration in genotype A/A was found when compared to G/G ($p = 0.0316$) (**Figure 1**). Reticulocytes, uric acid and bilirubin levels were not significantly different among the studied genotypes. There was no significant difference in hemolysis parameters for regions rs4671393 and rs1886868. HbF concentration was similar for three studied regions of the *BCL11A* (**Table 3**).

Regarding treatment, patients were stratified into two groups: those using HU at a dose lower than 10 mg/kg/day and patients on HU at a dose higher than or equal to 10 mg/kg/day. When compared, the hemolysis parameters varied according to HU dose, as a statistical increase in LDH concentration in patients taking HU at doses below 10 mg/kg/day was observed. Regarding time, there was a statistical decrease in reticulocyte count ($p = 0.0155$) in patients on HU for a period longer than 50 months. For other hemolysis parameters, results were similar (**Figure 2**).

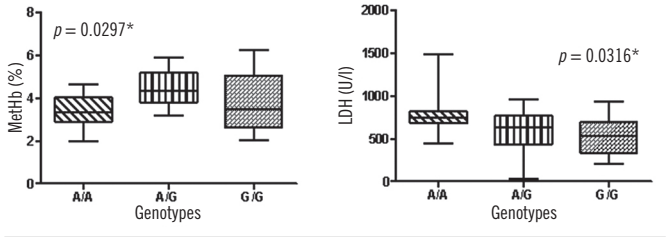

FIGURE 1 – *Hemolysis biomarkers according to* BCL11A *genotypes in region rs7557939*

The p*-values were obtained by one-way Anova with the Tukey post-hoc test. AA:* n = *11; A/G:* n = *20; GG:* n = *13; MetHb: significant difference between groups A/A and A/G, and LDH between groups A/A and G/G, which is significant at the 0.05 level. Anova: analysis of variance; MetHb: methemoglobin; LDH: lactate dehydrogenase.*

TABLE 3 – HbF concentration in the studied genotypes at the *BCL11A* gene

Polymorphisms	Genotypes (mean HbF)	p-value
rs7557939	A/A 11.66% A/G 13.36% G/G 14.44%	0.6978
rs4671393	A/A 11.27% A/G 14.24% G/G 13%	0.6529
rs11886868	C/C 14.73% C/T 13.78% T/T 10.95%	0.4321

The p*-value was obtained by one-way analysis of variance test (Anova). Anova: analysis of variance.*

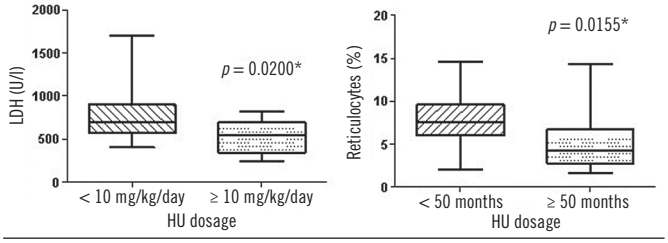

FIGURE 2 – *Hemolysis biomarkers according to use of HU*

Comparing means of the hemolysis parameters in patients with AF with HU < 10 mg/kg/day, n = *24; ≥ 10 mg/kg/day:* n = *21, and* n ≤ *50 months = 23,* n ≥ *50 months = 22. The* p*-value was obtained by an unpaired* t*-test and the Mann-Whitney test. HU dose calculated for an average weight of 70 kg per person, with* p < *0.05*; HU: hydroxyurea; LDH: lactate dehydrogenase.*

DISCUSSION

This study investigated the association between hemolysis biomarkers and *BCL11A* gene polymorphisms in SCD patients. As reported in the literature[6, 7, 9, 10, 19-25], we found a significant increase in reticulocyte count and serum measurements of MetHb, LDH, and total direct and indirect bilirubin in patients with SCD compared to those in the control group. The increase in these parameters was due to SCD, as the disease promotes chronic hemolysis.

Association of hemolysis biomarkers with *BCL11A* region polymorphisms in SCD patients is somewhat innovative, without any studies with this theme for comparison. The association of regions rs7557939, rs4671393, and rs11886868 of the *BCL11A* gene with hemolysis biomarkers showed a relationship of MetHb with genotypes in the rs7557939 region. MetHb concentration showed a significant increase in genotype A/G (heterozygous) compared to A/A (mutant).

We also found an association between LDH and the rs7557939 region, with a significant increase in genotype A/A (mutant) compared to G/G genotype (wild-type). Thus, MetHb and LDH biomarkers are associated with the region rs7557939 on the *BCL11A* gene in SCD adult patients at baseline. This may be related to decreased HbF genotypes, which can contribute to increased hemolysis parameters, and may suggest the involvement of the *BCL11A* gene polymorphism in this region as a modulator of HbF concentration, thus aggravating hemolysis balance in these patients. These results differ from those observed by Sheehan *et al*. (2013)[26], which showed an increase in the concentration of HbF in children with SCD, without using HU with a mutant genotype (A/A) for the rs7557939 region of *BCL11A*. This difference in results can be attributed to the fact that different patient populations have been studied, as well as SCD presenting a heterogeneous clinical picture attributed to multifactorial causes, such as age, drug use, and genetic and environmental components[27-29].

The study of Lettre *et al*. (2008)[13] showed that regions of polymorphisms rs7557939 and rs4671393 are more strongly associated with increased HbF than the rs11886868 region. However, other studies found an association between rs11886868 and rs4671393 regions, with a concentration of HbF[15, 27, 30]. The association of genotypes of the *BCL11A* gene with increased HbF was not observed in this study, what can be attributed to restricted sample size and HU use.

HU is the treatment for SCD to increase concentration of HbF, improving the clinical condition of patients. Patients using HU at its highest dose (10 mg/kg/day) showed a decrease in the LDH enzyme. The reduction in reticulocyte count was observed in patients on HU for longer than 50 months. These results may be related to the benefits of HU for patients, increasing the concentration of HbF and decreasing hemolysis. Apparently, the improvement is associated with time and dose; however, one must note that many factors are unknown about HU action in patients with SCD, such as optimal therapeutic dose, drug mechanism of action, intense variability of therapeutic response (for drug metabolization and the presence of genetic polymorphisms), and probable drug cytotoxicity and genotoxicity[13].

Several factors may influence concentration of HbF, such as haplotypes of the βS globin gene, polymorphisms in promoter regions of γ-globin, and use of HU. Pharmacogenetic studies are needed to analyze the influence of several SNPs in their concentration of HbF and the response to treatment with HU, such as *BCL11A*, *XmnI*, *ARG1*, *ARG2*, and the development of potential targeted therapies for SCD treatment[14]. This study is unprecedented in Northeastern Brazil, and formulates hypotheses that may aid in future research, including linkage disequilibrium.

CONCLUSION

The results showed that patients with SCD, even when taking HU, have elevated hemolysis parameters LDH and MetHb and may be associated in some way with the *BCL11A* gene region. Since this is an initial and innovative study, further research should be carried out with the objective of studying the role of the *BCL11A* gene in the clinical manifestations and consequent alterations of biomarkers in SCD.

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RESUMO

Introdução: *Pacientes com anemia falciforme (AF) apresentam hemólise crônica com biomarcadores séricos aumentados. Os polimorfismos genéticos do gene* BLC11A *modulam a hemoglobina fetal (HbF), reduzindo, assim, a hemólise.* ***Objetivo:*** *Associar os polimorfismos do gene* BCL11A *aos marcadores de hemólise: reticulócitos, bilirrubina, ácido úrico, lactato desidrogenase (LDH) e meta-hemoglobina (MetHb) em pacientes com AF.* ***Métodos:*** *O estudo incluiu 45 pacientes com AF que utilizavam hidroxiureia (HU), tratados em um hospital de Fortaleza, Ceará, Brasil, e 80 indivíduos saudáveis como grupo-controle. A dosagem de MetHb, ácido úrico e bilirrubina foi realizada por método espectrofotométrico; LDH, pelo método cinético; contagem de reticulócitos, pelo método manual; e avaliação de polimorfismos* BCL11A*, por reação em cadeia da polimerase (PCR) em tempo real. Os dados foram*

analisados usando o software *estatístico GraphPad Prism. O nível de significância foi < 5%.* ***Resultados:*** *Na região rs7557939 do gene* BCL11A*, o genótipo A/G mostrou aumento significativo de MetHb (*p = *0,0297), e o genótipo A/A esteve relacionado com a alta concentração de LDH (*p = *0,0316). Pacientes em uso de HU em doses ≥ 10 mg/kg/dia apresentaram diminuição de LDH (*p = *0,02), e o tratamento por mais de 50 meses foi relacionado com a contagem de reticulócitos (*p = *0,0155).* ***Conclusão:*** *Polimorfismos na região rs7557939 do gene* BCL11A *parecem interferir de alguma forma nas manifestações clínicas de pacientes com AF, o que sugere uma relação com a concentração de MetHb e LDH. Este estudo foi pioneiro na investigação da associação de biomarcadores de hemólise com polimorfismos do gene* BCL11A *na AF.*

Unitermos: *anemia falciforme; hemólise; marcadores biológicos; polimorfismo genético; hemoglobina fetal.*

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