The 844ins68 cystathionine beta-synthase and C677T MTHFR gene polymorphism and the vaso-occlusive event risk in sickle cell disease

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Abstract

Introduction: Sickle cell disease (SCD) is an inflammatory condition with an increase in the adhesion of sickled erythrocytes, and it is a potential cause of vaso-occlusive episodes, an event related to clinical manifestations, morbidity and mortality. The cystathionine beta-synthase enzyme gene (CBS) and the methylenetetrahydrofolate reductase enzyme gene (MTHFR) are risk factors for thromboembolic disorders. This study evaluated the frequency of the 844ins68 CBS and C677T MTHFR gene polymorphisms and their possibility to be risk factors for vaso-occlusive crises.

Material and methods: In total 91 blood samples from SCD patients were studied by PCR-RFLP and PCR-allele-specific, for the SCD genotype confirmation and polymorphism identification.

Results: The presence of clinical manifestations related to vaso-occlusive crises were more frequent among patients with the Hb SS genotype \( (p = 0.007) \). The CBS enzyme gene was three times more frequent \( (p = 0.011) \) among patients with vaso-occlusive complications. The MTHFR gene mutation frequency showed no increased risk for vaso-occlusive crises in SCD patients \( (p = 0.193) \). The interaction between the two polymorphisms was evaluated in 12.08% of the SCD patients and doubled the vaso-occlusive disease risk \( (\text{relative risk: } 2.16) \).

Conclusions: We conclude that the presence of 844ins68 CBS and C677T MTHFR gene polymorphism was a risk factor for vaso-occlusive episodes in the SCD patients evaluated.

Key words: sickle cell disease, polymorphism, cystathionine beta-synthase, methylenetetrahydrofolate reductase.

Introduction

As described in the literature, elevated levels of homocysteine (Hcy) are associated with a higher risk of cardiovascular diseases and venous thrombosis [1, 2]. However, the physiological mechanisms that explain this association are still not completely clear. Hcy levels can be genetic determined, but can also be affected by environmental factors, such as lifestyle, age, gender, nutrition, physical activity, and drugs or cigarettes. In most cases, an abnormal level of Hcy is not caused by one isolated factor; instead, it is frequently a result of a combination of factors [3]. Therefore, it is important to understand how, exactly, genetic factors are...
involved in the production of plasma Hcy. It is also important to promote the prevention and treatment of thrombotic diseases that result from high levels of homocysteine and how genetic mutations in the enzymes involved in the metabolism of Hcy, such as 844ins68 on the cystathionine-β-synthase (CBS) and C677T on the methylenetetrahydrofolate reductase (MTHFR), can contribute to higher levels of plasma Hcy [4-6].

The 844ins68 mutation on the CBS gene, in homozygous or heterozygous state, is considered an independent risk factor for artery occlusion. The mutation on the MTHFR gene also causes lower enzyme activity, which results in higher levels of serum Hcy [7]. Furthermore, MTHFR gene alterations increase the risk of vascular diseases and DNA hypomethylation, and can also affect and influence cell adhesion and nitric oxide activation [8]. Previous studies have stated that the co-existence of the genotype C677TT on the MTHFR gene and the 844ins68 variant on the CBS gene, are significant for patients with thrombosis of unknown origin. This finding suggests that the presence of these altered genotypes puts the individual at a risk of vaso-occlusive crises [9].

Sickle cell disease is an inflammatory condition in which mediator-enzymes activate the endothelial cells and increase the adhesion of platelets, white blood cells, and sickle-shaped red blood cells to the endothelium, which can lead to vaso-occlusive episodes [10]. The cloting of both deep and superficial veins is the most important factor in the sickle cell disease physiopathology, and can be used to explain some of the clinical manifestations, as well as the morbidity and mortality [11]. The aim of this study was to evaluate the frequency of 844ins68 on the CBS gene and C677T on the MTHFR gene polymorphisms and their correlation with vaso-occlusive episodes in patients with sickle cell disease from Brazil.

**Material and methods**

**Patients’ data**

This study was approved by the Ethical Committee from the UNESP-IBILCE and the Clinical Hospital from the Goiás Federal University (UFG).

Analyses were conducted for 91 sickle cell patients from the UFG Clinical Hospital, 51 women and 40 men aged 19-59 years. Forty-nine patients used Hydroxyurea. All the patients studied received a prophylactic treatment with folic acid of 5 mg/day since the SCD diagnosis. Through analysis of the patients’ records, five presented ischemic stroke, eleven experienced ischemic bone necrosis, five had priapism, four had malleolar ulcers, six presented osteomyelitis, six experienced complications during pregnancy, sixteen had retinopathy, two had alveolar hemorrhages, and four had splenic infarction. All of these vaso-occlusive complications were confirmed by the patient’s hematologist. There is no difference between male and female vaso-occlusive events except for the priapism in males.

All patients participated in the ambulatory evaluation and 5 ml of blood samples were collected with EDTA anticoagulants after filling a consent form. Patients were genotyped for the Sickle Cell Disease [12]. DNA was extracted from peripheral blood lymphocytes and isolated by phenol-chloroform methodology [13].

**Genotyping**

The mutations in the globin genes were performed by PCR-RFLP [14] and PCR-EA [15] to determine the sickle cell disease genotype (Hb SS, Hb SC or Hb S/Beta thal.). The CBS polymorphisms were detected using PCR as previously described [6]. The MTHFR polymorphisms were detected using PCR-RFLP [16]. The fragment amplified was digested using *Hinfl* Fast Enzyme Restriction® (Fermentas) and separated in 2% agar gel. These methods are able to detect all three possible genotypes for the globin gene, CBS and MTHFR polymorphism: homozygous wild type, heterozygous variant type and homozygous variant type. Figure 1 shows a picture of agarose gel for MTHFR polymorphism evaluated in SCD patients.

We applied the $\chi^2$ test with a level of significance of 90% ($p = 0.05$) and Yates’ correction at $n < 5$ to compare the polymorphism frequencies. Calculations for relative risk were made, and the $\chi^2$ test was used to verify the statistical significance. The calculations were made using the Statistica software, version 7.0.

**Results**

Of all 91 sickle cell disease patients evaluated, 48 presented the Hb SS genotype, 20 Hb SC, and...
23 Hb S/Beta Thalassemia (IVS 1-110). The correlation between the SCD genotypes and the occurrence of vaso-occlusive event was significant ($\chi^2 = 9.969; GL = 2; p = 0.007$), with the majority of the cases occurring in patients with the Hb SS genotype, as shown in Table I. The beta globin haplotypes evaluated showed prevalence of the Bantu/Benin heterozygous profile, as expected for the Brazilian population. Clinical observations and laboratory exams revealed that 33.33% of the SCD patients who received Hydroxyurea had a significant increase in Hb F concentration, despite the haplotype. This increase was more than 15% in relation to the initial treatment, particularly in Hb SS patients.

The heterozygous genotype of CBS gene polymorphism was found in 32.96% of the SCD patients, and the homozygous variant type was found in 1.09%. The heterozygous frequency of the CBS polymorphism was found in 32.96% of the SCD patients, and the homozygous variant type was found in 17.58%, and for the MTHFR variants it was 19.23%, neither to the MTHFR polymorphisms ($\chi^2 = 0.799; GL = 1; p = 0.371$).

Comparison between vaso-occlusive events and polymorphism allele frequency showed a significant difference for the CBS gene ($\chi^2 = 6.502; GL = 1; p = 0.011$). The SCD patients did not show evidence of major vaso-occlusive events, except for one with a vascular cerebral accident. This mutation was approximately three times more frequent in patients with clinical sickle cell complications (Table II). In the case of the MTHFR gene mutation, we did not find the same correlation ($\chi^2 = 1.698; GL = 1; p = 0.193$).

The interaction between the two polymorphisms studied was found in eleven patients (12.08%) and 91% of them presented vaso-occlusive episodes, which suggests a significant association that determines an increased risk of these clinical complications in these patients ($\chi^2 = 8.158; GL = 1; p = 0.004$). The combination between MTHFR and insertion of 86bp on the CBS gene showed a 100% increase in the relative risk (RR: 2.16; 95%) for vaso-occlusive episodes, arterial disease, and/or venous disease, with a confidence interval (CI) of 0.31-1.68, as shown in Table III.

**Discussion**

The clinical manifestations of sickle cell disease are more severe in Hb SS genotype [11]. The manifestations can also be influenced by the inheritance of the beta globin cluster haplotypes associated with Hb F concentration and differential gene expression with inhibitory effects on the sickling process. Patients with Hb F above 20% presented

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Table I. Presence or absence of vaso-occlusive events in relation to the globin gene genotypes

| Genotype      | Presence of vaso-occlusive events (n = 59) | Absence of vaso-occlusive events (n = 32) | N = 91 |
|---------------|------------------------------------------|------------------------------------------|-------|
| SS            | 34                                       | 14                                       | 48    |
| S/Beta Thal   | 8                                        | 15                                       | 23    |
| SC            | 12                                       | 8                                        | 20    |

$\chi^2$ – Chi-Square test, DF – degree of freedom, $p \leq 0.05$

Table II. Allele frequency for the CBS and MTHFR polymorphisms compared to the presence or absence of the vaso-occlusive event in SCD patients (n = 91)

| Genes | Allele number | Allele frequency | Allele number | Allele frequency | $\chi^2$ | DF | p      |
|-------|---------------|------------------|---------------|------------------|----------|----|--------|
| MTHFR | W             | 92               | 0.78          | W                | 55       | 0.86| 1.698  |
|       | M             | 26               | 0.22          | M                | 9        | 0.14| 0.193  |
| CBS   | W             | 91               | 0.77          | M                | 55       | 0.92| 6.502  |
|       | M             | 27               | 0.23          | M                | 5        | 0.08| 0.011* |

$\chi^2$ – Chi-Square test, DF – degree of freedom, $p \leq 0.05$
mild clinical manifestations [17]. In this study, SCD patients who received Hydroxyurea confirm the results of the literature that fewer transfusions are needed when they receive Hydroxyurea treatment, despite the haplotypes inherited, mainly because of the admixture of the Brazilian populations.

The relation between the presence of the insertion of 68pb on the CBS gene and the vaso-occlusive events was significant in the SCD patients studied. The literature reports that this mutation is a risk factor for venous thromboembolism and premature atherosclerotic disease in other groups of patients, but not for sickle cell patients [6]. The interaction between the two polymorphisms evaluated in this study, which were inherited together, was associated with a 100% increased risk of developing a vaso-occlusive episode in SCD patients as supported by the literature [18, 19].

The frequency of the MTHFR polymorphism in SCD patients did not show an increased risk of a vaso-occlusive event and confirms findings of Morielli et al. [18] concerning the Brazilian population, but contradicts the literature about other similar populations [7, 8]. It is important to note that studies suggest that these polymorphisms, which affect folate metabolism and which are risk factors for vaso-occlusive patients, have different effects within different populations, with evidence of a gene-gene and gene-nutrient interaction [19]. The literature shows that 200 µg of folic acid a day can reduce the concentration of homocysteine by up to 60%, and a daily dose of 400 µg is associated with an up to 90% reduction in the concentration of homocysteine [18]. Folic acid can be a prophylactic therapy for those patients with polymorphisms on the MTHFR gene. It lowers homocysteine levels, and, consequently, the occurrence of vaso-occlusive complications like thrombosis and cerebral vascular accidents. The lack of vaso-occlusive complications in these SCD patients can be attributed to therapies involving folic acid.

Although the majority of these SCD patients do not present serious vaso-occlusive events, unobserved complications are the leading cause of chronic organ and tissue damage. However, the frequency of clinically detectable vaso-occlusive crises should not be the unique parameter for evaluating the gravity of sickle cell disease, and it cannot serve as a reliable parameter for effective therapy in every patient. When we consider the shortage of studies involving Brazilians of African descent related to the frequency and interaction of the genetic polymorphisms evaluated, we conclude that the presence of the 844ins68 mutation on the CBS gene is a risk factor for vaso-occlusive episodes in SCD patients. The results obtained will contribute to improvement of the understanding of the clinical manifestations experienced by these patients, and will also offer more information to the use of prophylactic treatments and certain therapies.

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