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

Maza Alves Jacob[1], Celso da Cunha Bastos[2], Claudia Regina Bonini-Domingos[1]

[1]Biology Department UNESP, São José do Rio Preto, São Paulo, Brazil
[2]Federal University of Goiás (UFG) School of Medicine, Clinical Hospital, Goiânia, Goiás, Brazil

Submitted: 29 December 2009
Accepted: 8 July 2010

Arch Med Sci 2011; 7, 1: 97-101
DOI: 10.5114/aoms.2011.20611

Corresponding author:
Claudia Regina Bonini-
-Domingos
Biology Department, UNESP
Rua Cristóvão Colombo n 2265
Bairro Jardim Nazareth
CEP: 15054-000
São José do Rio Preto
São Paulo, Brazil
E-mail: bonini@ibilce.unesp.br

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 (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 clotting 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.

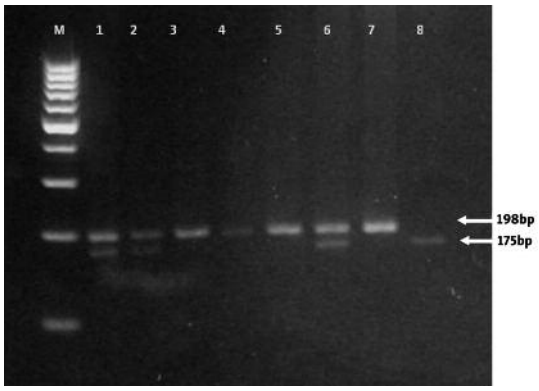

Figure 1. Agarose gel of MTHFR polymorphism evaluated in SCD patients. Samples 1, 2 and 6 show a heterozygote profile. Polymorphism analysis of the MTHFR allele: *Hinfl*-digest PCR fragments (175 bp) and the normal allele (198 bp)
M – molecular weight markers

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 χ^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 χ^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 (χ^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 shown 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 MTHFR gene polymorphism was found in 34.06%, and the homozygous variant type was found in 2.19%. The allele frequency of CBS variants was 17.58%, and for the MTHFR variants it was 19.23%, also in Hardy-Weinberg equilibrium. We observed a significant difference in the CBS polymorphism presence between Hb SS and Hb S/Beta thal. genotypes (χ^2 = 5.219; GL = 1; p = 0.022). However, when hemoglobin genotypes and the presence of polymorphisms were evaluated together, we did not observe a significant difference in the allele CBS variant frequency (χ^2 = 3.441; GL = 1; p = 0.064), neither to the MTHFR polymorphisms (χ^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 (χ^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 (χ^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 (χ^2 = 8.158; GL = 1; p = 0.004). The combination between MTHFR and insertion of 86pb 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

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
χ^2	χ^2 = 9.969; DF = 2; p = 0.007		
χ^2		χ^2 = 8.380; DF = 8; p = 0.015	

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

Table III. MTHFR and CBS polymorphism interaction and risk factor for the vaso-occlusive events in SCD patients (n = 49)

MTHFR/CBS interaction	Vaso-occlusive event		Total
	Yes	No	
Presence	10	1	11
Absence	16	22	38
Total	26	23	49

Chi-Square test (χ^2 = 8.158, GL = 1, 95% IC: 0.31-1.68, p = 0.004)

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	Presence of the vaso-occlusive event (n = 59)				Absence of the vaso-occlusive event (n = 32)				χ^2	DF	p
	Allele number		Allele frequency		Allele number		Allele frequency				
	W	M	W	M	W	M	W	M			
MTHFR	92	26	0.78	0.22	55	9	0.86	0.14	1.698	1	0.193
CBS	91	27	0.77	0.23	59	5	0.92	0.08	6.502	1	**0.011***

χ^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 arteriosclerotic 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.

Acknowledgments

The authors would like to thank the CNPq for the financial support; all the patients for their kind collaboration; the healthcare professionals from the UFG Clinical Hospital who enabled the research. We would also like to thank Heury Sousa Ferreira and Henderson Vinícius de Souza for help with the statistical analyses and Danille Deremo for the English version.

References

1. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ 2002; 25: 1202-10.
2. Den Heijer M, Lewington S, Clarke R. Homocysteine, MTHFR and risk of venous thrombosis: A meta-analysis of published epidemiological studies. J Thromb Haemost 2005; 3: 292-9.
3. Schneede J, Refsum H, Ueland PM. Biological and environmental of plasma homocysteine. Semin Thromb Hemost 2000; 6: 263-79.
4. Eldibany MM, Caprini JA. Hyperhomocysteinemia and thrombosis. Arch Pathol Lab Med 2007; 131: 872-84.
5. Andreassi MG, Botto N, Cocci F, et al. Methylenetetrahydrofolate reductase gene C677T polymorphism, homocysteine, vitamin B12, and DNA damage in coronary artery disease. Hum Genet 2003; 112: 171-7.
6. Tsai MY, Bignell M, Schwichtenberg K, Hanson NQ. High prevalence of a mutation in the cystathionine beta-synthase gene. Am J Hum Genet 199; 59: 1262-7.
7. Shmeleva VM, Kapustin SI, Papayan LP, Sobczyńska-Malefora A, Harrington DJ, Savidge GF. Prevalence of hyperhomocysteinemia and the MTHFRC677T polymorpism in patients with arterial and venous thrombosis from North Western Russia. Thromb Res 2003; 111: 351-8.
8. Juo SHH, Liao YC, Kuo CL, et al. The MTHFR 677 C/T polymorphism influences plasma levels of adhesion molecules and nitric oxide. Thromb Res 2008; 121: 549-54.
9. Gaustadnes M, Rudiger N, Rasmussen K, Ingerslev J. Intermediate and severe hyperhomocysteinemia with thrombosis: A study of genetic determinants. Thromb Haemost 2000; 83: 554-8.
10. Walter PB, Fung EB, Killilea DW, et al. Oxidative stress and inflammation in iron-overloaded patients with beta thalassaemia or sickle cell disease. Br J Haematol 2006; 135: 254-63.
11. Serjeant GR. The emerging understanding of sickle cell disease. Br J Haematol 2001; 112: 3-18.
12. Bonini-Domingos CR. Métodos laboratoriais para o diagnóstico de hemoglobinopatias e talassemias [Spanish]. São José do Rio Preto, HN 2006; 121.
13. Pena SDJ. DNA Bioprints: simple non-isotopic DNA fingerprints with biotinnylated probes. Electrophoresis 1991; 12: 146-52.
14. Sayki Y, Ueno I, Nakakoshi R, et al. Differentiation of selected abnormal hemoglobin by PCR-RFLP method. Rinsho Byori 1997; 45: 1163-6.
15. Chinelato-Fernandes AR. Diferenciação molecular de variants de hemoglobinas no Brasil [Spanish]. Sao Jose

do Rio Preto, 227f. Thesis (Ph.D. in Genetics) – Institute of Biosciences, Letters and Exact Sciences, University Estadual Paulista 2003; 227f.
16. Frosst P, Blom Hj, Milos R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Natur Genet 1995; 10: 111-3.
17. Steinberg MH, Lu ZH, Barton FB, et al. Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. Blood 1997; 89: 1078-88.
18. Morelli VM, Lourenco DM, D'almeida V, et al. Hyperhomocysteinemia increases the risk of venous thrombosis independent of the C677T mutation of the methylenetetrahydrofolate reductase gene in selected Brazilian patients. Blood Coagul Fibrinolysis 2002; 13: 271-5.
19. Devlin AM, Clarke R, Birks J, et al. Interactions among polymorphisms in folate-metabolizing genes and serum total homocysteine concentrations in a healthy elderly population. Am J Clin Nutr 2006; 83: 708-13.