

Genetic polymorphisms and cerebrovascular disease in children with sickle cell anemia from Rio de Janeiro, Brazil

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ABSTRACT

The aim of the present work was to examine possible genetic risk factors related to the occurrence of cerebrovascular disease (CVD) in Brazilian population, the frequency of β^S -globin gene haplotypes and co-inheritance with α -thalassemia ($-\alpha^{3.7kb}$) and single nucleotide polymorphism of methylenetetrahydrofolate reductase (MTHFR-C677T), Factor V Leiden (FV-G1691A) and prothrombin (PT-G20210A) genes in children from Rio de Janeiro. Ninety four children with sickle cell anemia (SCA) were included, 24 patients with cerebrovascular involvement and 70 patients without CVD as control group. The mean age of children at the time of the cerebrovascular event was similar to the control group. The frequency of $-\alpha^{3.7kb}$ thalassemia was similar in both groups ($p=0.751$). Children with Bantu/Atypical β^S -globin gene haplotype presented 15 times more chance (OR=15.4 CI 95% 2.9-81.6) of CVD than the other β^S -globin gene haplotypes. The C677T polymorphism of MTHFR gene was similar in both groups ($p=0.085$). No mutation in the FV Leiden or PT genes was found. A large study seems necessary to establish the role of these genetic polymorphisms in Brazilian miscegenated population.

Key words: sickle cell anemia, cerebrovascular disease, alpha-thalassemia, beta-globin haplotypes, genetic polymorphism.

Polimorfismos genéticos e doença cerebrovascular em crianças com anemia falciforme do Rio do Janeiro, Brasil

RESUMO

Avaliar o papel da talassemia alfa ($-\alpha^{3.7kb}$), dos haplótipos da globina β^S , e mutações nos genes da metileno-tetrahidrofolato redutase (MTHFR-C677T), fator V de Leiden (FV-G1691A) e protrombina (PT-G20210A) como fatores de risco para a doença cerebrovascular em pacientes com anemia falciforme. Foi realizado um estudo de caso controle com 94 crianças portadoras de anemia falciforme, 24 com doença cerebrovascular (DCV) e 70 sem DCV como grupo controle. A frequência de talassemia $-\alpha^{3.7kb}$ foi semelhante em ambos os grupos ($p=0,751$). Crianças portadoras do haplótipo Bantu/Atípico da globina β^S apresentam 15 vezes mais chances de desenvolverem DCV (OR=15,4 IC 95% 2,9-81,6) do que os outros haplótipos. A frequência do polimorfismo MTHFR-C677T foi semelhante em ambos os grupos ($p=0,085$) e não foi observada mutação nos genes fator V e protrombina. Estudos com maior número de casos são necessários para esclarecer o papel desses polimorfismos genéticos na nossa população.

Palavras-chave: anemia falciforme, acidente vascular cerebral, talassemia alfa, globinas beta, polimorfismo genético.

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Sickle cell anemia (SCA) is a genetic disorder caused by homozygosity for a single β -globin gene mutation ($\beta^6\text{GAG}\rightarrow\text{GTG}$), in which glutamic acid has been substituted for valine at the sixth codon of β -globin chain. Despite this fact, the clinical course of patients suffering from SCA is extremely variable, the severity of manifestations ranging from asymptomatic to a very severe course^{1,2}. The phenotypic variability maybe explained by some genetic factors, those related to globin genes have been well recognized². There is evidence that SCA and other chronic hemolytic anemia are characterized by a hypercoagulable state with increased of thrombin and fibrin generation as well as platelet activation with an augmented risk for thromboembolic complications³.

Cerebrovascular disease (CVD) is a major complication of sickle cell disease⁴. Stroke is a very severe event and is estimated that approximately 11% of patients will have a clinical stroke by age 20^{2,4}, and 21% have evidence of silent infarction on magnetic resonance imaging⁵. CVD is more frequent among patients with SCA than patients with sickle cell disease, and approximately 10% of children between the ages of 2 to 16 years are at risk for stroke^{4,6}, the incidence is higher in the 1 to 9 years⁴.

Anemia, high leukocytes count, high blood pressure and acute chest syndrome are risk factors for the development of CVD in sickle cell disease². A familial predisposition to stroke has been observed, suggesting that genetic factors may contribute to CVD risk⁷. Fetal hemoglobin (HbF) and β^S -globin haplotypes are the most studied genetic modulator for SCA, but the diversity of the disease is not completely explained by this modulation, and several potential genetic modifiers have been studied. These candidate genes include mediators of inflammation, vaso-regulation, blood coagulation, haemostasis, growth factors, cytokines and cytokine receptors, and transcriptional factors⁸. Furthermore, the C677T polymorphism in methylenetetrahydrofolate reductase gene as well as Factor V Leiden (FV-G1691A) and prothrombin (PT-G20210A) have been implicated in vascular diseases⁸. Despite the recent studies indicating probable genetic factors, only few have been recognized to influence the risk of stroke in SCA¹.

To examine possible genetic risk factors related to the occurrence of CVD in a group of Brazilian patients, the frequency of β^S -globin gene haplotypes and co-inheritance with α -thalassemia ($-\alpha^{3.7\text{kb}}$) and single nucleotide polymorphism in methylenetetrahydrofolate reductase (MTHFR-C677T), Factor V Leiden (FV-G1691A) and prothrombin (PT-G20210A) genes were determined in children with SCA followed at the Instituto Estadual de Hematologia Arthur de Siqueira Cavalcanti, Rio de Janeiro.

METHOD

After review by local Institutional Ethics Boards and informed consent signed by parent or guardian, blood samples from 94 children with SCA were collected and genomic DNA was extracted from peripheral blood mononuclear cells using a commercial kit (Puregene[®] DNA Purification System-Gentra). Twenty four children were randomly selected from a cohort of patients that have had CVD, and 70 children were randomly selected from clinic records of the neonatal hemoglobinopathy screening program of the state of Rio de Janeiro, Brazil. The children without CVD were matched by sex and closest in age at the time of the cerebrovascular event to the index cases. Cerebrovascular disease was defined by the presence of: [a] stroke with resultant ischemia and neurologic symptoms or signs for more than 24 hours plus abnormal brain computed tomography or magnetic resonance imaging, or [b] transient ischemic attacks for less than 24 hours plus abnormal brain computed tomography or magnetic resonance imaging, or [c] transcranial Doppler with highest time-averaged mean of the maximum velocity in middle cerebral artery, distal internal carotid artery, anterior cerebral artery and bifurcation, velocities greater than 200 cm/sec, according to the STOP trial^{6,9}.

The β^S -globin gene cluster haplotype (Bantu or CAR [Central African Republic] Benin, Senegal, Arab-Indian and Atypical) was determined by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). A pattern of six polymorphic restriction sites was identified within the beta-globin gene complex (5' γ^G , γ^C , γ^A , $\psi\beta$, 3' $\psi\beta$, 5' β). Products were digested with restriction endonucleases for polymorphisms identification (*XmnI* [5' γ^G], *HindIII* [γ^C], *HindIII* [γ^A], *HincII* [$\psi\beta$], *HincII* [3' $\psi\beta$], *HinfI* [5' β]), according to Sutton et al.¹⁰.

The α -thalassemia ($-\alpha^{3.7\text{kb}}$) single gene deletion was detected by PCR, primers were designed to amplify the junction fragments of the α -thalassemia determinants that could be easily identified by size. Since of the $-\alpha^{3.7\text{kb}}$ deletions partially remove both α -genes, the positive amplification was used to indicate heterozygosity when a deleted allele was also present¹¹. The C677T mutation in the MTHFR gene, G1691A in FV Leiden and G21210A in prothrombin gene were determined by PCR-RFLP according to the methods described by Frosst et al.¹², Zöller and Dahlbäck.¹³ and Poort et al.¹⁴, respectively. The MTHFR C677T mutation, primers generate a fragment of 198 bp, the substitution creates a *Hinf I* recognition sequence which digests the 198 bp fragment into 175 and 23 bp fragments. The region in exon 10 of the factor V gene was amplified the 161 bp amplicon obtained was subjected to *MnlI* digestion, which produced frag-

ments of 43 and 118 bp. A 345-bp fragment from exon 14 and the 3'-UT region of the prothrombin gene was amplified. A new *Hind* III site (-A/AGCTT-) was introduced in the amplified fragments from the less-frequent allele (A₂:AAG) yielding two fragments (322 bp and 23 pb in length) after enzyme digestion. The more frequent allele (A₁:GAG) lacks the restriction site and therefore generates only a 345-bp fragment by PCR-*Hind*III digestion.

Analyses were performed using SPSS (Statistics Package for Social Science). Comparison of categorical data was done by chi-square test, including odds ratios (OR) and 95% confidence interval (CI), t-student test was used to compare age mean between the groups and p values ≤0.05 were considered significant.

RESULTS

Ninety four children (48 boys and 46 girls) were included, with a mean age of 6.6 years (range from 3.2 to 15 years), 24 (13 boys and 11 girls) patients with cerebrovascular involvement were participating in regular blood transfusion program, and 70 (35 boys and 35 girls) patients with normal transcranial Doppler and no CVD. The mean age of children at the time of the diagnosis of CVD was 5.1 years (±SD 1.4 years) and of 5.4 years (±SD 1.6 years) for the control group (p=0.53).

The frequency of α-thalassemia (-α^{3.7kb}) in the group with cerebrovascular disease was of 15.8% (3/19) compared to 22.4% (15/67) in the controls (p=0.751).

Table shows molecular characteristics of children according to the presence of CVD. In eight patients,

α-chain fragment was not amplified, and in four out of these eight, the β^S-globin haplotype could not be genotyped, probably due to DNA quality.

The number of children with CVD was higher (χ²=16.8 and p=0.001) among those with Bantu/Atypical than the ones with Bantu/Bantu and Bantu/Benin haplotypes (Table). Children with Bantu/Atypical haplotype presented 15 times more chance (OR=15.4 [CI 95% 2.9-81.6]) of cerebrovascular involvement than the other β^S-globin gene haplotypes (Table).

The C677T polymorphism of MTHFR gene was found in 37.5% (9/24) of children with CVD and in 20% (14/70) of the control group (χ²=2.9 and p=0.085). This polymorphism was found in 23% of patients (23/94) and, four children were homozygous for the mutation, while 15 were heterozygous. None of the four homozygous children presented cerebrovascular involvement. No mutation in the FV Leiden or PT genes was found.

DISCUSSION

Stroke is a catastrophic event and represents the second (~10%) leading cause of death in both adult and children⁴. During the last decades, several attempts have been made to detect genetic modifiers of the clinical course in SCA^{1,2}, as well as genetic polymorphisms implicated in the occurrence of cerebrovascular disease. Despite all the effort, very few genes have been related to the clinical course of SCA. This report represents the first study of genetic factors and CVD in a group of Brazilian SCA patients.

Table. Molecular characteristics of patients according to the presence of cerebrovascular disease.

Molecular characteristics	Cerebrovascular disease		Total	χ ²	p
	Present n (%)	Absent n (%)			
β ^S -globin haplotype				15.4	0.001 [#]
Bantu/Bantu	11 (50)	36 (52.9)	47		
Bantu/Atypical	7 (31.8)	2 (2.9)	9		
Bantu/Benin	4 (18.2)	30 (44.1)	34		
Total	22	68*	90*		
-α ^{3.7kb} thalassemia				0.38	0.751
Present	3 (15.8)	15 (22.4)	18		
Absent	16 (84.2)	52 (77.6)	68		
Total	19	67	86**		
MTHFR C677T				2.96	0.085
Present	9 (37.5)	14 (20)	23		
Absent	15 (62.5)	56 (80)	71		
Total	24	70	94		

χ²Pearson Chi-square; [#]Bantu/Atypical versus Bantu/Bantu and Bantu/Benin; *In four patients β^S-globin haplotype was not done due to degraded DNA; **Eight patients were not typed for α-thalassemia due to degraded DNA; MTHFR: methylenetetrahydrofolate reductase.

Several studies showed that the incidence of CVD for SCA patients with α -thalassemia is lower than that for patients without α -thalassemia^{4,15,16}.

In our results, the frequency of thalassemia was lower among patients with CVD, but with no significant difference when compared to the ones without cerebrovascular involvement. Furthermore, only 15.8% had α -thalassemia compared to the reported prevalence of 20 to 25% in Afro-Brazilian population with SCA¹⁷.

An increased HbF concentration can ameliorate SCA severity. Individual HbF variation is largely genetically controlled, with at one side caused by mutations of the globin gene complex, named hereditary persistence of fetal hemoglobin and at the other side, recently identified three major quantitative trait loci (QTLs). The first known QTL is the *Xmn1-HBG2* site at the gamma-globin gene, and now joined by two other loci: *HBS1L-MYB* intergenic region 6q and *BCL11A*. Common polymorphisms at the three QTLs strongly affect HbF levels in healthy individuals and patients with hemoglobinopathies and account for relatively large proportion (20-50%) of HbF levels variation¹⁸. SCA patients have HbF levels ranging from 1 to 30%, concerning the role of β^S -globin haplotypes on clinical severity. Carriers of the Senegal or Arab-Indian haplotypes have the highest HbF levels and a mild clinical course, and individuals with Bantu haplotypes the lowest HbF levels⁸.

Regarding β^S -globin haplotypes and cerebrovascular disease the studies have been conflicting. Sarnaik and Ballas¹⁵ reported that the presence of at least one Bantu and/or atypical haplotype increases the risk for CVD. Kinney et al.¹⁹ described that silent cerebral infarcts in SCA were related to Senegal haplotype. On the other hand, Bernaudin et al.²⁰ reported no influence of β^S -globin haplotypes on the presence of abnormal high cerebral velocities by transcranial Doppler in patients with SCA. Adorno et al.²¹ in Salvador, Brazil, described in 5 cases with CVD the presence of at least one Bantu haplotype. Our results suggest that the presence of Bantu/Atypical haplotype is a risk factor for CVD.

The role of hyperhomocysteinemia as a potential risk for cerebrovascular disease is still controversial²². The most common polymorphism associated with raised homocysteine level is the C-to-T substitution at nucleotide 677 of the gene methylenetetrahydrofolate reductase, and the relationship of this mutation with vascular diseases have been described in some population²³. This study failed to demonstrate a positive relationship between the presence of C677T MTHFR polymorphism and CVD in SCA patients. Same results have been reported by Cumming et al.²⁴. Furthermore, caution is needed in the asserting that this genetic polymorphism played a role in the occurrence of cerebrovascular disease

in SCA, as none of cerebrovascular patients presented homozygosity for the MTHFR polymorphism.

According to previous report factor V Leiden (G1691A) mutation was found at high frequency in Caucasian (5.27%), and lower prevalence in Hispanic (2.21%) and Afro-Americans (1.23%)²⁵. The prothrombin (G20210A) mutation is present in 1.1% of Caucasian Americans and in a very low frequency in Afro-descendants (0.3%)²⁶. Both genetic polymorphisms are rare in Afro-descendants. In Brazil, reports described a low frequency in general population, ranging from 1-2% for FV-G1691A and 0.7-3.6% for PT-G20210A²⁷⁻²⁹. Only two papers described the frequency of these polymorphisms in SCD. Andrade et al.²⁷ found a frequency of 2.73% for both genes mutation (2 out of 73 patients), and Couto²⁹ et al. failed to find PT-G20210A variant in SCA patients from the Northeast of Brazil. The difference observed between these reports maybe due to the origin of patients, in São Paulo the significant contribution of European immigrants in the population formation may explain these findings. It is necessary a large study, including in different Brazilian regions to reach a more precise prevalence of these polymorphisms.

This is the first genetic study carried out in children with SCA and cerebrovascular involvement in Brazil, a large study seems necessary to establish the real role of these genetic polymorphisms in our very miscegenated population.

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