MTHFR Polymorphic Variant C677T Is Associated to Vascular Complications in Sickle-Cell Disease

Betânia L.D. Hatzlhofer,[1,2] Marcos André C. Bezerra,[3]
Magnun N.N. Santos,[4] Dulcinéia M. Albuquerque,[5] Elizabete M. Freitas,[6]
Fernando F. Costa,[5] Aderson S. Araújo,[1] and Maria Tereza C. Muniz[6,7]

Vaso-occlusion is a determinant for most signs and symptoms of sickle-cell anemia (SCA). The mechanisms involved in the pathogenesis of vascular complications in SCA remain unclear. It is known that genetic polymorphisms associated with thrombophilia may be potential modifiers of clinical features of SCA. The genetic polymorphisms C677T and A1298C relating to the enzyme methylenetetrahydrofolate reductase (MTHFR), a clotting Factor V Leiden mutation (1691G→A substitution of Factor V Leiden), and the mutant prothrombin 20210A allele were analyzed in this study. The aim was to find possible correlations with vascular complications and thrombophilia markers in a group of SCA patients in Pernambuco, Brazil. The study included 277 SCA patients, divided into two groups: one consisting of 177 nonconsanguineous SCA patients who presented vascular manifestations of stroke, avascular necrosis, leg ulcers, priapism, and acute chest syndrome (group 1); and the other consisting of 100 SCA patients without any reported vascular complication (group 2). Molecular tests were done using either polymerase chain reaction (PCR) restriction fragment length polymorphism or allele-specific PCR techniques. Comparisons between the groups were made using the χ^2 test. The 677 CT and TT genotypes showed a significant risk of vascular complications ($p = 0.015$). No significant associations between the groups were found when samples were analyzed for the *MTHFR* A1298C allele ($p = 0.913$), Factor V G1691 ($p = 0.555$), or prothrombin G20210A mutation ($p = 1.000$). The polymorphism *MTHFR* C677T seemed to be possibly predictive for the development of some vascular complications in SCA patients among this population.

Introduction

SICKLE-CELL ANEMIA (SCA) is a monogenic disorder derived from a single point mutation, a substitution (GAG→GTG) at position six of the β-globin gene located in chromosome 11. However, it presents a multigenic phenotype with several complications, and different modulators dictate the individual variations of the disease. Some contributing factors for this phenotypic variability are already well established, such as the coexistence of α thalassemia, β-globin gene haplotypes, and Hb F levels. Unfortunately, none of them are able to clarify the different phenotypic expression observed in patients (Nagel and Steinberg, 2001; Steinberg, 2005).

In Brazil, the β^S gene distribution is very heterogeneous, ranging from 2% to 10%, following the proportion of African descendants in the different geographical regions of the country. For instance, the frequency of the β^S gene in the general population of Pernambuco, a state located in the northeastern region of Brazil, is 4% (Cançado and Jesus, 2007). Patients with SCA in Pernambuco tend to have a severe clinical course, poor prognosis, and frequent hospitalizations due to vaso-occlusive crises and higher mortality among children in their first year of life. In this state, 79.2% of SCA patients are homozygous for the Central African Republic β^S haplotype, which has been associated with low levels of Hb F and clinical features of greater severity (Steinberg, 2005; Bezerra *et al.*, 2007). Acute chest syndrome (ACS), avascular necrosis (AVN) of the femoral head, leg ulcers, stroke, and priapism are the most frequent and important vascular complications in our patients.

[1]Centro de Hematologia e Hemoterapia de Pernambuco—HEMOPE, Recife, Pernambuco, Brasil.
[2]Faculdade de Ciências Médicas, Universidade de Pernambuco, Recife, Pernambuco, Brasil.
[3]Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.
[4]Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas-UNICAMP, Campinas, São Paulo, Brasil.
[5]Hospital de Hematologia e Hemoterapia, Universidade Estadual de Campinas-UNICAMP, Campinas, São Paulo, Brasil.
[6]Instituto de Ciências Biológicas-ICB, Universidade de Pernambuco-UPE, Recife, Pernambuco, Brasil.
[7]Centro de Oncohematologia Pediátrica-CEONHPE/UPE, Hospital Universitário Oswaldo Cruz, Recife, Pernambuco, Brasil.

Adhesion of sickle cells to the endothelium plays a very important role in vaso-occlusion phenomena. These are the determining physiopathological events that cause most of the signs and symptoms of sickle-cell disease (Frenette and Atweh, 2007; Kaul *et al.*, 2009).

Thrombosis is also an important aspect of the clinical spectrum of sickle-cell disease. Hemostatic abnormalities have been observed in SCA patients, such as reduced levels of natural anticoagulant inhibitors and increased thrombin production, platelet activation, and circulating tissue factor levels. In summary, almost all procoagulant or anticoagulant components become altered in sickle-cell disease. These findings show that the steady state of sickle-cell disease is a hypercoagulable state (Bayazit and Kilinç, 2001; Ataga and Orringer, 2003; Al-Absi *et al.*, 2006; Akinyoola *et al.*, 2008).

Genetic mutations have been identified as a cause of thrombophilia in different populations. The molecular basis for hereditary thrombophilia includes mutations in Factor V, prothrombin, and the methylenetetrahydrofolate reductase (*MTHFR*) gene (Slavik *et al.*, 2009).

The C677T variant of the *MTHFR* gene has been correlated with a 50% decrease in the activity of the key enzyme in homocysteine (Hcy) metabolism (MTHFR) and consequent increases in plasma Hcy levels, which may cause damage to endothelial cells. A second *MTHFR* polymorphism (A1298C) results in mildly decreased MTHFR activity, but does not elevate plasma Hcy (Al-Absi *et al.*, 2006). Hyperhomocysteinemia is considered to be a risk factor for several thrombotic events, including the cerebral and peripheral types, as well as coronary disease (Engbersen *et al.*, 1995; Khan and Dickerman, 2006). However, there are several studies providing evidence that the mutations G1691A on Factor V and G20210A on the prothrombin gene are associated with an elevated risk of deep vein thrombosis and with the main causes of hereditary thrombophilia (Kordes *et al.*, 2002; Khan and Dickerman, 2006; Ercan *et al.*, 2008).

Because of the evidence of increased activation of the coagulation system in SCA, it is important to evaluate the role of genetic variants relating to thrombophilia in vascular complications of SCA. The aim of this study was thus to investigate possible associations of the *MTHFR* variants C677T and A1298C, Factor V Leiden mutation, and prothrombin mutation with vascular complications in sickle-cell disease.

Patients and Methods

Sample

Two hundred seventy-seven patients with Hb SS and Hb Sβ[0] thalassemia were enrolled for this study. The patients were divided in two groups. Group 1 consisted of 177 nonconsanguineous SCA patients who had important vascular complications such as stroke, AVN, leg ulcers, priapism, and ACS. These conditions were confirmed by means of transcranial Doppler and X-rays, and the clinical records of ACS and priapism were taken from patients' medical files. Group 2 consisted of 100 nonconsanguineous adult SCA patients without previous stroke, AVN, leg ulcers, priapism, or ACS. Patients on hydroxyurea and chronic transfusion therapy were not included in this group, as their use would have a protective effect against vascular complications (Orah and Platt, 2008).

This study was approved by the Research Ethics Committee of HEMOPE, and an informed consent statement was signed by each study participant.

Methods

The sickle-cell disease was diagnosed by means of high-performance liquid chromatography (Bio Rad, Hercules, CA). DNA was extracted from peripheral blood leukocytes using the phenol–chloroform technique (Lahiri and Nurnberger, 1991).

The *MTHFR* C677T polymorphism was detected by means of polymerase chain reaction (PCR) restriction fragment length polymorphism, using the forward primer 5′-TGAAG GAGAAGGTGTCTGCGG-3′ and the reverse primer 5′-AG GACGGTGCGGTGAGAGTG-3′. The C→T transition creates a restriction site for the *Hin*fI enzyme, and the digested product was isolated through gel electrophoresis in 3% agarose prepared with ethidium bromide. The fragments were viewed under UV light (Frosst *et al.*, 1995). The homozygote for the wild-type allele (CC) produces a 198-bp fragment; the heterozygote (CT) produces 198-, 175-, and 23-bp fragments; and the mutant homozygote (TT) produces 175- and 23-bp fragments. The *MTHFR* A1298C polymorphism was detected by means of allele-specific PCR, using primers for separate amplification of the wild-type and mutant alleles (allele A: forward 5′-GGAGCTGACCAGTGAAGA-3′ and reverse 5′-TGTGACCATTCCGGTTTG-3′ amplified a 77-bp fragment; allele C: forward 5′-CTTTGGGGAGCTGAAGGA-3′ and reverse 5′-AAGACTTCAAAGACACTTG-3′ amplified a 120-bp fragment) (Biselli *et al.*, 2008).

Factor V Leiden mutation G1691A was detected through amplification of exon 10 of the Factor V gene, by means of PCR with the forward primer 5′-CTTGAAGGAAATGCCC CATTA-3′ and the reverse primer 5′-TGCCCAGTGCTTAA CAAGACCA-3′, followed by digestion of the PCR product using *Mnl*I endonuclease (Andrade *et al.*, 1998). The G20210A fragment on the mutant prothrombin gene was amplified through PCR using primers as described by Poort *et al.* (1996), with 5′-TCTAGAAACAGTTGCCTGGC-3′ as the forward primer, and 5′-ATAGCACTGGGAGCATTGAAGC-3′ as the reverse primer. The amplified 345-bp fragment was digested using *Hin*dIII endonuclease, giving a 322-bp fragment in the presence of the mutant allele (20210A). The *Hin*dIII- and *Mnl*I-digested products were viewed under UV light by means of electrophoresis on 3% agarose gels (Andrade *et al.*, 1998). The products from digestion of the G20210A region of the mutant prothrombin gene and the G1691A region of the Factor V gene were separated through electrophoresis on 3% agarose gels, and were then prepared using ethidium bromide and viewed under UV light (Arsov *et al.*, 2006).

Statistical analysis

The χ^2 test was first used to evaluate whether the groups were in the Hardy–Weinberg equilibrium. Fisher's exact test and the χ^2 test were used for genotype and allele frequency comparisons. The risk was defined in terms of odds ratios (ORs) with confidence intervals (CIs), with significance levels up to 95%. In all statistical evaluations, $p < 0.05$ was considered significant. Software STATA/SE 9.0 and Microsoft® Office Excel (2007) were used for data analysis.

Results

Among the 277 patients studied, 124 (44.7%) were female, and 153 (55.2%) were male; 262 (94.6%) had Hb SS and 15 (5.4%) had Sβ[0] thalassemia. Out of the 177 patients in the

TABLE 1. DEMOGRAPHIC CHARACTERISTICS
AND DISTRIBUTION OF DIFFERENT TYPES OF VASCULAR
COMPLICATIONS FOUND IN SICKLE-CELL DISEASE PATIENTS

	Group 1	Group 2
Number of patients	177 (63.9%)	100 (36.1%)
Mean age in years (with range)	26.5 (5–72)	26.2 (18–58)
Sex		
Male	108 (61.0%)	45 (45%)
Female	69 (39.0%)	55 (55%)
Vascular complications:		
Stroke	45 (25.4%)	N
AVN	43 (24.3%)	N
ACS	31 (17.5%)	N
Priapism	34 (31.5%)[a]	N
Leg ulcers	67 (37.8%)	N
Combined complications	43 (24.3%)[b]	N

N, no vascular complications.
[a]31.5% of males presented priapism.
[b]Four cases associated between vascular complications and thrombosis.
ACS, acute chest syndrome; AVN, avascular necrosis.

group 1, who presented some form of vascular manifestation, 170 had a diagnosis of Hb SS and seven Sβ⁰ of thalassemia; out of the 100 patients in the group 2, 92 were diagnosed with Hb SS and eight with Sβ⁰ thalassemia. The clinical characteristics, age, and sex distribution observed in the two both groups can be seen in Table 1. The frequency of male patients was higher in the group 1 than in the group 2 ($p=0.0119$; OR 1.913).

The genotype distributions in the group 1 ($p=0.7704$) and in the group 2 ($p=0.9537$) were found to be in the Hardy–Weinberg equilibrium. The frequency of the *MTHFR* 677T allele was higher in the group 1 (50.8%) than in the group 2 (35.0%), and this difference was statistically significant ($p=0.015$; OR 1.9212; CI: 1.1587–3.1855) (Table 2), but when each complication is considered separately and on groups of one, two, and three complications and compared with the group 2 (without complications), no significant association with the *MTHFR* C677T polymorphism was observed (Table 3).

Analysis on the *MTHFR* C677T and *MTHFR* A1298C haplotype distribution showed higher frequency of the double mutant 677T/1298C in the group 1 patients (26; 15.2%) than in the group 2 (9; 9.1%), but without statistical significance ($p=0.1920$). The *MTHFR* A1298C, prothrombin mutation G20210A, and Factor V Leiden mutation analysis did not show any association with vascular complications, even when each complication was separately considered or on groups of one, two, and three complications. Homozygous mutations for Factor V Leiden and mutant prothrombin were not detected in any of samples (Table 2).

Molecular analysis on all of the 277 patients with sickle-cell disease showed that five patients (1.80%) had the heterozygous genotype for the G20210A prothrombin mutation, and three patients (1.08%) had the heterozygous form for the G1691A Factor V Leiden mutation. There were 15 patients (5.41%) with the homozygous form (TT) for the *MTHFR* C677T polymorphism, as well as 110 patients (39.7%) with the heterozygous form (CT), such that the total prevalence of the 677T allele was 45.1%. *MTHFR* A1298C polymorphism analysis was performed on 269 patients, and there were 11 patients (4.0%) with the homozygote genotype for the mutant allele, as well as 99 (36.8%) with the heterozygote genotype. Thus, the frequency of the 1298C mutant allele was 40.9%.

Discussion

The Hematology Hospital of the HEMOPE Foundation is the reference institution for diagnosis and treatment of sickle-cell disease in Pernambuco; around 1800 patients are currently being followed up. Priapism, AVN, stroke, leg ulcers, and ACS are frequently found manifestations in patients attended at HEMOPE, and thus there is a great need for studies on these complications in this region. Identification of genetic variants that may be associated with vaso-occlusion is important for better understanding of this complex condition.

The present study ascertained that the difference between the observed frequencies of the *MTHFR* 677T allele in the group 1 (50.8%) and the group 2 (35.0%) was statistically significant ($p=0.015$). Therefore, the *MTHFR* C677T polymorphism can be considered to be a likely predisposing factor for the development of vascular complications among the

TABLE 2. ASSOCIATIONS PRESENTED BY *MTHFR* C677T, *MTHFR* A1298C, FACTOR V LEIDEN,
AND PROTHROMBIN G20210A WITH THE RISK OF VASCULAR COMPLICATIONS IN SICKLE-CELL DISEASE

Genotype	Group 1 (%)	Group 2 (%)	OR	95% CI	p
MTHFR 677	n = 177	n = 100			
CC	87 (49.1%)	65 (65.0%)	1	Reference	0.015[a]
CT/TT	90 (50.8%)	35 (35.0%)	1.9212	1.1587–3.1855	
MTHFR 1298	n = 171	n = 98			
AA	102 (59.6%)	57 (58.1%)	1	Reference	0.913[a]
AC/CC	69 (40.3%)	41 (41.8%)	0.9405	0.5679–1.5574	
Factor V Leiden	n = 177	n = 100			
GG	174 (98.3%)	100 (100%)	—	—	0.555[b]
GA	3 (1.69%)	0 (0%)	—	—	
Mutant prothrombin	n = 177	n = 100			
GG	174 (98.3%)	98 (98.0%)	1	Reference	1.000[b]
GA	3 (1.69%)	2 (2.00%)	0.8448	0.1388–5.1431	

[a]χ^2 test.
[b]Fisher exact test.
MTHFR, methylenetetrahydrofolate reductase; CI, confidence interval; OR, odds ratio.

Table 3. Vascular Complications Acute Chest Syndrome, Stroke, Avascular Necrosis, Leg Ulcers, Priapism, and Combined Complications Associated with the *MTHFR* C677T

	MTHFR 677		
	CC n (%)	CT/TT n (%)	p
Vascular complications			
Stroke	26 (57.8)	19 (42.2)	0.518
AVN	22 (51.2)	21 (48.8)	0.171
ACS	15 (48.4)	16 (51.6)	0.148
Priapism	12 (35.3)	22 (64.7)	0.098
Leg ulcers	34 (50.7)	33 (49.3)	0.094
Combined complications			
No complications	65 (65.0)	35 (35.0)	0.058
1 complication	67 (48.9)	70 (51.1)	
2 complications	18 (48.6)	19 (51.4)	
3 complications	2 (66.7)	1 (33.3)	

sickle-cell disease patients studied. A similar result was found by Moreira Neto *et al.*, when studying 53 SCD patients (HbSS and HbSC) in southeastern Brazil. They found greater presence of the *MTHFR* 677T allele among SCA patients who presented vascular complications (Moreira Neto *et al.*, 2006). Kutlar *et al.* found a statistically significant association between the *MTHFR* C677T polymorphism and AVN among American patients with SCA. They found that the frequency of the 677T allele in patients diagnosed with AVN was 35.6%, whereas it was 12.9% for this mutation in patients not presenting the complication ($p = 0.006$). These authors suggested that this polymorphism could be a possible predisposing factor for this vascular problem (Kutlar *et al.*, 2001). These results contradicted what had been found in previous studies, such as the ones by Andrade *et al.* in Brazil and Zimmerman and Ware in the United States. Neither of these studies found any association between the *MTHFR* C677T polymorphism and vascular complications in SCA cases (Andrade *et al.*, 1998; Zimmerman and Ware, 1998). However, these studies were performed with small samples. Adekile studied sickle-cell disease patients (HbSS and Sβ thalassemia) in Kuwait, and also did not ascertain any association. This author suggested that a larger number of patients would be needed to confirm the result (Adekile, 2001). A more recent study by Al-Saqladi *et al.* on 102 children (HbSS) in Yemen did not find any association between the *MTHFR* C677T polymorphism and the severity of the disease. However, the frequency of this polymorphism has been found to be quite low in most populations (7.35%) (Al-Saqladi *et al.*, 2010). In our study, we also analyzed separately the vascular complications: stroke, AVN, ACS, priapism, leg ulcers, and groups of one, two, and three complications, and we did not find a significant association with the *MTHFR* C677T polymorphism. We suggested this could be happen because of the small number of samples when they are divided.

The presence of the 677T allele is associated with increased plasma Hcy levels, and this increase is a risk that was previously established by vessel thrombosis and arteriosclerosis (Slavik *et al.*, 2009). Hcy levels were not evaluated in our study, but Lowenthal *et al.* showed that the plasma Hcy levels in SCA patients were significantly higher than in healthy control patients. This was somewhat unexpected, since sickle-cell patients make continuous use of folic acid to improve their erythropoiesis, and the increased folate intake induces a decrease in plasma Hcy levels (Lowenthal *et al.*, 2000). In the study by van der Djis *et al.* (1998), sickle-cell patients who used oral folic acid had subclinical folate levels compared with control patients diagnosed with Hb AA.

It is likely that the folate levels necessary for normalization of the plasma Hcy levels in SCA patients are higher than in healthy people, since these patients have a higher nutritional folic acid need than seen in the general population. In the present study, it was observed that patients carrying the 677T allele presented a higher risk of vascular complications, and these patients probably need even greater folate oral supplementation, because of the presence of the allele.

The *MTHFR* A1298C polymorphism has previously been associated with cardiovascular disease, kidney disease, spina bifida, and hypercoagulability disorders, including stroke (Volcik *et al.*, 2000; Haviv *et al.*, 2002). Our sickle-cell disease patient population showed a frequency of 40.9% for the *MTHFR* 1298C allele. Robien and Ulrich (2003) described the prevalence of *MTHFR* A1298C within different populations around the world: the frequency of the C allele varies from 32% to 34% in Asia and from 46% to 62% in Eastern Europe, and in Africans, a frequency of 38.5% has been found. Al-Absi *et al.* (2006) compared 106 SCA patients with healthy controls in Bahrain, and found a frequency of 62% for *MTHFR* 1298C, which was higher in patients than in controls (47%).

This is the first study to investigate the *MTHFR* A1298C polymorphism and vascular complications in sickle-cell disease. However, the presence of the mutant allele did not present a risk of vascular complications from the disease ($p = 0.913$). Even when we analyzed the combination of *MTHFR* A1298C and *MTHFR* C677T, comparing the groups 1 and 2, there was no statistical significance ($p = 0.1920$). The *MTHFR* C677T single-nucleotide polymorphism is located in exon 4 of the *MTHFR* gene and results in a thermolabile protein with decreased enzymatic activity, whereas the *MTHFR* A1298C polymorphism occurs in a regulatory region of the MTHFR enzyme (exon 7) and is associated with decreased enzyme activity, but not with thermolability (Castro *et al.*, 2004; Reeves *et al.*, 2009).

Factor V Leiden and mutant prothrombin are considered to be the main hereditary risk factors for venous thrombosis (Fawaz *et al.*, 2004). The relative risk of venous thrombosis increases threefold to eightfold and twofold to threefold, respectively (Poort *et al.*, 1996; Ramos *et al.*, 2006). However, these mutations were not considered to be risk factors for vascular complications from sickle-cell disease in the present study, and this result is in accordance with other studies in the literature (Andrade *et al.*, 1998; Ramos *et al.*, 2006).

The frequency of heterozygous genotypes for Factor V Leiden in Israelis, Arabs, Canadians, and Indians ranges from 1% to 8.5%, and some European studies have reported frequencies of 5% to 8%. However, the prevalences found in Greece, Sweden, and Lebanon have been much higher, reaching up to 15% in some areas. On the other hand, the mutation is rare in black Africans and apparently not present in Chinese and Japanese people (Ridker *et al.*, 1998). In our study population, a frequency of 1.08% was found for the Factor V Leiden G1691A mutation, a low frequency, similar to afro-descendants. Arruda *et al.* (1997) found a similar prevalence of this mutation (2%) in a Brazilian population.

A study conducted in 11 centers in Europe found prevalences of the prothrombin G20210A mutation of 0.7% to 4%. Similarly to Factor V Leiden, this mutation has been found to be extremely rare in nonwhite populations (black or Asian people) (Zivelin *et al.*, 1998). In our study, we found a prevalence of 1.80% for the prothrombin G20210A mutation.

Because of the low frequency within any given population, the exact mechanism for Factor V Leiden and mutant prothrombin as risk factors for vascular complications in SCA patients can only be clarified through studies with higher numbers of patients in different populations around the world.

In this study, we found that the *MTHFR* C677T polymorphism was associated with the development of vascular complications in sickle-cell disease. Our results are in agreement with previous studies in southeastern Brazil and the United States (Kutlar *et al.*, 2001; Moreira Neto *et al.*, 2006), but not with studies in other regions of Brazil and elsewhere in the world (Andrade *et al.*, 1998; Zimmerman and Ware, 1998; Adekile, 2001). This is possibly because of the different genetic compositions of these populations. In Brazil, divergent results may occur because of the high degree of racial miscegenation in our population.

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Address correspondence to:
Maria Tereza Cartaxo Muniz, Ph.D.
Centro de Oncohematologia Pediátrica
Hospital Universitário Oswaldo Cruz
310 Arnóbio Marques St.
50100-130 Santo Amaro
Recife
Pernambuco
Brasil

E-mail: tcartaxo.upe@gmail.com
maria.muniz@upe.pe.gov.br

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