Angiotensinogen M235T Gene Polymorphism is a Genetic Determinant of Cerebrovascular and Cardiopulmonary Morbidity in Adolescents with Sickle Cell Disease

Mohsen Saleh ElAlfy, MD,* Fatma Soliman Elsayed Ebeid, MD,*,
Tarek Mostafa Kamal, MD,† Deena Samir Eissa, MD,‡
Eman Abdel Rahman Ismail, MD,‡ and Samar Hassan Mohamed, MB, BCh*

Background: Cerebrovascular stroke is a common critical complication of sickle cell disease (SCD). Angiotensinogen (*AGT*) M235T gene polymorphism is associated with risk of ischemic stroke and cardiovascular disease. *Aim:* We investigated the potential association between angiotensinogen M235T gene polymorphism and susceptibility to cerebrovascular and cardiopulmonary complications in adolescents with SCD. *Methods:* Forty-six patients with SCD in steady state were studied stressing on history of stroke, hydroxyurea/chelation therapy, hematological profile, and echocardiographic findings. Polymerase chain reaction-based restriction fragment length polymorphism analysis was used to detect *AGT* M235T gene polymorphism. Fifty sex- and age-matched healthy controls were enrolled for assessment of M235T gene polymorphism pattern. *Results:* The distribution of *AGT* M235T gene polymorphism was similar between SCD patients and healthy controls. The frequency of T allele of *AGT* M235T gene polymorphism (TT and MT genotypes) was significantly higher among patients with history of manifest stroke ($P < .001$). Patients with TT and MT genotypes had higher incidence of cardiopulmonary complications ($P = .041$) as well as higher percentage of HbS ($P < .001$) and lower hemoglobin level ($P = .008$) compared with those with MM genotype. Serum ferritin, liver iron concentration, and cardiac T2* were not related to T alleles or genotypes. Logistic regression analysis revealed that M235T genotype was a significant independent factor related to the occurrence of stroke among patients with SCD (Odds Ratio 14.05, 95% confidence interval 3.82-28.91; $P = .001$). *Conclusion: AGT* M235T gene polymorphism may represent a genetic modifier to vascular morbidities in Egyptian patients with SCD.

Key Words: Sickle cell disease—angiotensinogen M235T gene polymorphism—stroke—cardiopulomonary complications

From the *Pediatrics Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt; †Medical Genetics Pediatric Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt; and ‡Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

Received July 16, 2018; revision received October 9, 2018; accepted October 13, 2018.

Conflict of interest: Nothing to declare.

The manuscript has not been submitted elsewhere nor previously published.

The corresponding author, on behalf of all authors, hereby states that all authors have contributed to the manuscript in significant ways, have reviewed and agreed upon the manuscript content.

Address correspondence to Fatma Soliman ElSayed Ebeid, 2 Lotfy Elsayed st, Ain Shams University staff's campus, Cairo, Egypt. E-mailes: elalfyms@hotmail.com, dr.fatma_ebeid@yahoo.com, kamaltmgen@yahoo.com, dr.deena_samir@yahoo.com, eman.ismail_70@yahoo.com, samarmohamedagt@yahoo.com.

1052-3057/$ - see front matter

https://doi.org/10.1016/j.jstrokecerebrovasdis.2018.10.019

Introduction

Sickle cell anemia (SCA) is an inherited autosomal recessive blood disorder due to mutations in the normal β globin gene[1] in which, the red blood cells (RBCs) are rigid, sticky and can lodge in small blood vessels, which leading to slow or blockage in the blood flow and decrease in oxygen delivery to body tissues.[2] Sickle cell disease (SCD) is remarkable for its clinical heterogeneity, even among individuals with identical genotypes. Some individuals experience morbidity and mortality in early childhood, while others have a relatively mild course, and normal or near normal life expectancy.[3] Cerebrovascular stroke is one of the most common critical complications of children with SCD with an incidence of .61 to .76 per 100 patients per year in the first 20 years of life, which is 300 times higher than that observed in children without SCD (.0023 per 100 patients per year).[4-7] SCD-associated stroke is responsible for 20% of mortality among SCD patients.[8]

Understanding the genetics underlying the heritable sub-phenotypes of SCA would be prognostically useful, could inform personalized therapeutics, and might help the discovery of new "druggable" pathophysiologic targets. Genotype-phenotype association studies have been used to identify novel genetic modifiers associated with development of specific SCD related complications.[3,9] Consequently, the stroke sub-phenotype of SCA could be heritable and genetically modifiable.[10] Currently, there are no known genetic or molecular risk factors to predict the susceptibility of stroke in patients with SCD. However, there are candidate genes and genetic modifiers for increasing susceptibility to stroke including those implicated in endothelial cell inflammation and adhesion.[11,12]

Fetal hemoglobin concentration and coincident α-thalassemia are the major modulators of the phenotype of disease, but are unlikely to be the only ones.[13-16] These variables do not explain all of the clinical heterogeneity of SCD.[3] The presence of α-thalassemia may reduce risk of stroke in SCD.[17-19] However, the relation between HbF and stroke, increased transcranial Doppler velocity or silent infarction may be equivocal and no evidence of a protective effect in infants with SCD but there is some evidence of protection in adults.[17,20,21] Other genetic modifiers include multiple gene such as *VCAM1*, *ILR4*, *ADBR2*, *HLA*, and *LDLR*,[19,22] but few have been validated.[9]

It has been reported that the presence of relative hypertension is considered a risk factor for stroke in patients with SCD.[23] The renin angiotensin system (RAS) is one of the major control systems for blood pressure and sodium homeostasis. These actions are coordinated through integrated actions in the kidney, cardiovascular system, and the central nervous system.[24] Angiotensinogen (*AGT*) is a key component of the RAS.[25,26] The *AGT* M235T gene polymorphism is a single base pair substitution of thymine (T) with cytosine (C) in the nucleotide 704 (T704C) at exon 2 of the angiotensinogen gene localized at chromosome 1q42-43, resulting in the substitution of methionine with threonine in amino acid position 235 at the pre-proangiotensinogen molecule (M235T). In addition, T235 alleles consider the mutant allele, while M235 alleles consider the wild type.[27]

AGT M235T gene polymorphism has been linked to essential hypertension,[28-30] ischemic stroke,[31,32] risk of myocardial infarction,[33,34] hypertrophic cardiomyopathy,[35] and renal disease.[36,37] The current study aimed to investigate the potential association between *AGT* M235T gene polymorphism and susceptibility to cerebrovascular and cardiopulmonary complications in adolescents with SCD.

Subjects and Methods

Participants

This case-control study included 46 patients with SCD aged 12-18 years who were randomly recruited from the regular attendants of the Pediatric Hematology Clinic, Pediatrics Hospital, Ain Shams University over a 1-year period after fulfilling inclusion criteria. Patients were diagnosed with SCD based on complete blood picture, reticulocyte count and markers of hemolysis as well as hemoglobin analysis using high performance liquid chromatography (HPLC) and confirmed by genotyping based on identification of β-globin gene mutations by polymerase chain reaction and subsequent reverse-hybridization to immobilized allele-specific biotinylated oligonucleotide probes covering the most common Mediterranean mutations.[38,39]

Patients with comorbidities that may be unrelated to SCD; infection, chronic inflammatory condition other than SCD, renal or cardiac disease, rheumatoid arthritis or other autoimmune diseases, hypothyroidism, diabetes mellitus, or steroid therapy were excluded. Some of these comorbidities may be associated with SCD or occur independently and therefore, we excluded them to avoid any confounding factors when studying the association between SCD and *AGT* M235T gene polymorphism and report the relation in a steady state SCD.

Fifty age-and sex-matched healthy individuals with no obvious medical disorder who were proven to be healthy after full clinical examination and laboratory investigations and not receiving any medication served as a control group for assessment of M235T polymorphism pattern in the healthy Egyptian population. This group was recruited from the same region as the case subjects; most were classmates or acquaintances of the case subjects. The clinical and laboratory data of patients and controls are listed in Table 1.

The details of the study design and laboratory investigations were explained to all individuals and/ or their parents, and informed consent was obtained from each patient, control, or their legal guardians before enrolment in the study. The procedures applied in this study were approved by the Ethical Committee of Human

Experimentation of Ain Shams University, and are in accordance with the Helsinki Declaration of 1975.

All included patients were subjected to detailed medical history that obtained by reviewing the patients filing system and by direct interviewing the patients and their guardian with special emphasis on demographic characteristics, smoking habits, family history of anemia and consanguinity, and history of vaso-occlusive crisis or acute chest syndrome. Transfusion history and treatment with hydroxyurea, iron chelation therapy, and any concomitant medications were obtained. The transfusion received was calculated as the transfusion index: volume of transfused packed red cells in ml per kg body weight per year (expressed as the mean value of the last three years). History of stroke and the presenting clinical manifestation, investigations and treatment given were recorded.

Thorough clinical examination was performed laying stress on full neurological assessment. Blood pressure measurements were obtained after 5 minutes of complete physical and mental rest; three reading were recorded then the average was calculated and plotted on the percentiles. The percentile then categorized into; normal blood pressure percentile (<90th), prehypertensive percentile (≥90th-≤95th) and hypertensive percentile (≥95th).[40]

Screening for pulmonary hypertension and cardiovascular abnormalities was performed by the non-invasive echocardiography with different modalities (Doppler, 2D imaging and M-mode) using Vivid E9 (GE Healthcare, Oslo, Norway) to evaluate left ventricular function, pulmonary artery pressure, tricuspid regurgitant jet velocity (TRV). SCD patients with cardiopulmonary morbidity include those with pulmonary hypertension risk and/or systolic left ventricle (LV) dysfunction. A TRV ≥2.5 m/s was used as a proxy for patients at risk for pulmonary hypertension.[41,42] Systolic LV dysfunction was defined by LV shortening fraction <30% or LV ejection fraction <55%.[43]

All SCD patients underwent magnetic resonance imaging (MRI) examination with a 1.5-T scanner (Philips-Intera, Holland) Achieva MR Unit without any contrast material using a 12-element phased array coil. Measurements of live iron concentration (LIC) were conducted by acquiring eight consequent T2* values and assessing T2* decay. Liver T2* values were converted into LIC values using the calibration curve.[44] For the measurement of myocardial T2*, a single short 20 seconds breath-hold axis mid-ventricular slice was acquired at eight simultaneously acquired echo times (TEs 1.4-13.6 ms/echospacing 1.6 ms). The myocardial T2* was calculated using the same method as that in the liver.[45]

Peripheral blood samples were withdrawn prior to blood transfusion and collected on potassium-ethylene diamine tetra-acetic acid (K2-EDTA) (1.2 mg/mL) as an anticoagulant for complete blood count (CBC), hemoglobin analysis, and genotyping. For chemical analysis, clotted samples were obtained and serum was separated by centrifugation for 15 minutes at 1000 × g.

Laboratory investigations included CBC using Sysmex XT-1800i (Sysmex, Kobe, Japan), examination of Leishman-stained smears for differential white blood cell (WBC) count, hemoglobin analysis by HPLC using D-10 (BioRad, Marnes La Coquette, France). Liver and kidney function tests and markers of hemolysis (lactate dehydrogenase [LDH] and indirect bilirubin) were performed on Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany). Serum ferritin was performed on Immulite 1000 analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). Serum ferritin level was measured at the start of the study with calculation of the mean value of the last year prior to the study in order to know the ferritin trend.

Genotyping of AGT M235T Gene Polymorphism

Genotyping was performed for SCD patients and healthy controls as follow: DNA was extracted from peripheral blood lymphocytes by spin column method of Gene JET™ Genomic DNA purification kit (#K072, Pure

Table 1. *Clinical and laboratory data and distribution of angiotensinogen M235T polymorphism in patients with sickle cell disease and healthy controls*

Variable		Patients ($n = 46$)	Controls ($n = 50$)	P value
Age (years)		15.9 ± 6.2	15.1 ± 5.4	.512
Males		23 (50%)	23 (46%)	.628
BMI (kg/m^2)		18.2 ± 4.0	19.8 ± 5.3	.127
WBC count (×10^9/L)		12.5 ± 4.6	8.1 ± 2.8	<.001
Hemoglobin (g/dL)		7.0 ± 1.5	12.7 ± 3.5	<.001
Lactate dehydrogenase (IU/L)		650.5 ± 187.3	285.1 ± 80.4	<.001
Indirect bilirubin (mg/dL)		1.98 ± 0.61	.35 ± .17	<.001
Genotypes	MM	13 (28.3%)	13 (26%)	.418
	MT	23 (50%)	28 (56%)	
	TT	10 (21.7%)	9 (18%)	
Alleles	M	49 (49%)	54 (54%)	.327
	T	43 (43%)	46 (46%)	

Abbreviations: BMI, body mass index; WBC, white blood cell. Data were expressed as mean ± SD where Student's *t*-test was used for comparisons or as number (percentage) where the chi-square (χ^2) test was used.

Extreme Fermentas Life Sciences, Thermo Scientific, Vilnius, Lithuania). Selection and synthesis of oligonucleotides was genotyped according to the published nucleotide sequences, and gene bank database of *AGT* gene polymorphisms.[27] Primers used to generate AGT region harboring M235T transition polymorphic locus were forward primer 5′CTTGGGGAGCTGAAGGACTACTAC3′ and reverse primer 5′CACTTTGTGACCATTCCGGTTTG3′.

The PCR program (S24 thermal cycler, Quanta Biotech-England, UK) was performed according to manufacturing manual and yielded an amplified PCR fragment of 165 bp in length for the fragment harboring *AGT* M235T gene polymorphism. Restriction enzyme digestion was performed and was analyzed using polymerase chain reaction-based restriction fragment length polymorphism. Wild type allele (235MM) yielded one fragment of 165 bp, while the polymorphic mutated homozygous variant (235TT) appeared as two fragments of 141 bp and 24 bp. The heterozygote (M235T) produced three fragments of 141 bp, 24 bp, and 165 bp.[27] The genotypes of the PCR products were determined by electrophoresis on 2% high resolution agarose gels stained with ethidium bromide in 1xTris-EDTA-Borate buffer against 50 bp ladder molecular weight Gene Ruler 50 bp DNA ladder (Fermentas, #SM0373, Thermo Scientific/Fermentas, Vilnius, Lithuania). The PCR products documented by Gel Documentation System and Software for DNA analysis (In Genius Syngene, UK) and the distribution of genotypes and allele frequencies were all statistically compared in patients versus healthy controls.

Statistical Analysis

Statistical analysis was done through IBM software (IBM Corporation, Armonk, NY). Kolmogrov-Smirnov test was used to examine the normal distribution of variables. Quantitative variables with normal distribution were presented as means and standard deviation. Variables with skewed distribution were presented as median and interquartile range (IQR; 75th and 25th percentiles). Comparison between two independent groups with quantitative data and parametric distribution was done by using Independent *t*-test. Categorical variables were described as number and percent and compared using chi-square (χ^2) test. The χ^2 test was also used to assess deviation of genotype distribution from Hardy-Weinberg equilibrium. Logistic regression analysis was used to define risk factors for stroke. Pearson correlation coefficients were used to assess the relation between two studied parameters. The confidence interval was set to 95% and consequently the *P*-value was considered significant at the level of $<.05$.

Results

Characteristics of the Study Population

The mean age for SCD patients was 15.9 ± 6.2 years and their median (IQR) age at diagnosis was 1 (.71-3.7) years. They included 22 patients with SCA, 23 with sickle β-thalassemia and one patient had sickle hemoglobin C disease. All included subjects were Caucasian originally from Egypt. Two patients (4.3%) were smokers. Family history of SCD was positive in 28 (60.9%) patients and 31 (67.4%) had positive parental consanguinity.

Mean systolic blood pressure was 106.9 ± 9.7 mmHg while mean diastolic blood pressure was 67.8 ± 7.5 mmHg. Most of the patients (96%) had normal (<90th) blood pressure, one patient (2.1%) was prehypertensive (≥90th-≤95th) and only one patient was hypertensive (≥95th).

According to the aforementioned definition of pulmonary hypertension and LV systolic dysfunction, pulmonary hypertension risk (elevated TRV ≥2.5 m/s) was present in 17.4% of the patients ($n = 8$) while LV systolic dysfunction was present in three (6.5%) patients (one of them had pulmonary hypertension risk as well). The overall percentage of patients who had cardiopulmonary complications (pulmonary hypertension risk and/or LV systolic dysfunction) was 23.9%.

Six patients with SCA experienced attack of ischemic stroke and none had hemorrhagic stroke. At the time of stroke, headache and dysarthria were the presenting symptoms in three of those patients while generalized tonic clonic convulsions were the presenting symptoms in two patients. Three patients had upper and lower limb weakness affecting the left side in two of them and the right side in one patient. All the six patients had confirmed radiological diagnosis of stroke and all had lacunar infarctions (watershed infarctions). Two (33.3%) patients had single attack of stroke and four (66.7%) had recurrent stroke. At the time of assessment, SCD who experienced a history stroke had no functional neurological deficit. They had lower hemoglobin level and higher HbS values compared with those without history of stoke.

All patients with SCD received blood transfusion; 39 patients were on regular transfusion, the 6 patients with history of stroke received blood exchanges which started therapy after they developed stroke and 1 patient was not regularly transfused. Twenty-four (52.2%) patients were on regular chelation and 35 (76%) patients received regular hydroxyurea with a dose ranged between 10 and 33 mg/kg/day but only 21 of them (60%) were complaint to hydroxyurea. Median (IQR) duration of hydroxyurea therapy was 5 (2-9) years. Concomitant medications used were folic acid, L-carnitine and phenoxymethyl penicillin.

AGT M235T Gene Polymorphism among the Study Population

The distribution of *AGT* M235T gene polymorphism was similar between SCD patients and healthy controls (Table 1). Comparison between patients who had homozygous (TT) or heterozygous (MT) mutant genotype and those who had wild type (MM) (Table 2) showed higher incidence of cardiopulmonary complications ($P = .041$) as

well as higher percentage of HbS ($P < .001$) and lower hemoglobin level ($P = .008$) in patients with T allele of *AGT* M235T gene polymorphism compared with those with MM genotype. Serum ferritin, liver iron concentration, and cardiac T2* were not related to T alleles or genotypes.

The frequency of T allele of *AGT* M235T gene polymorphism (TT and MT genotypes) was significantly higher among patients with history of manifest stroke where five of six patients who experienced stroke had TT genotype and one patient had MT genotype ($P < .001$) (Fig 1 and Table 2).

Logistic regression analysis was performed with the occurrence of stroke as the dependent variable and included all the significant variables between the two groups with and without history of stroke. Logistic regression analysis (Table 3) revealed that M235T genotype was a significant independent factor related to the occurrence of stroke among patients with SCD (odds ratio 14.05, 95% confidence interval 3.82-28.91; $P = .001$).

Discussion

In our study, six patients with SCA (12%) had pervious manifest ischemic stroke as confirmed by cerebral imaging. This highlighted the importance of regular clinical assessment including neurological examination in SCD patients. Globally, stroke affects up to 17% of patients with SCD.[46] Ischemic stroke is the most common type caused via interruption of the blood supply, whereas hemorrhagic stroke is much rarer.[47] Ischemic strokes disproportionately affect the youngest and oldest patients with SCD, while hemorrhagic strokes have the highest prevalence in patients between the ages of 20 and 29 years with SCD.[48]

All the patients who had history of stroke in this study started regular blood transfusion in form of manual blood exchange and hydroxyurea. In spite of the implementation of these secondary preventive measures for them, recurrence of stroke has occurred in 66.7% of patients. Chronic transfusion therapy (CTT) has long been used to prevent secondary ischemic strokes through adjusting the levels of hemoglobin and HbS.[48,49] Recurrent stroke in SCD can reach up to 60-80% within 3 years following the first stroke if therapy is not engaged [46,50] but with CTT, that risk is reduced to 10-20%. In a retrospective review of 137 pediatric patients treated at 14 centers with CTT due to a history of ischemic stroke, 22% of the patients had a recurrent stroke (2.2 per 100 person-years).[50] It has been reported that vasculopathy and silent infarcts, when present in patients who also have a history of acute stroke, can progress despite optimal transfusion.[51,52] Children with more severe vasculopathy at initiation of CTT appear to

Table 2. *Clinicopathological and radiological characteristics of the studied patients with sickle cell disease in relation to angiotensinogen M235T polymorphism*

Variable	TT & MT genotype ($n = 33$)	MM genotype ($n = 13$)	*P*-value
Age (years)	15.4 ± 4.2	16.5 ± 5.3	.523
Males, *n* (%)	16 (48.5)	7 (53.8)	.426
BMI (kg/m^2)	18.9 ± 4.15	17.6 ± 4.1	.371
Systolic BP (mmHg)	107.6 ± 11.2	106.1 ± 9.2	.529
Diastolic BP (mmHg)	69.8 ± 8.1	65.7 ± 7.3	.213
Delayed puberty, *n* (%)	9 (27.3)	4 30.7)	.618
Transfusion index (mL/kg/year)	240 (120-240)	185 (89-240)	.425
Transfusion iron input (mg/kg/day)	0.32 ± 0.12	.31 ± .13	.738
History of sickling crisis >3/year, *n* (%)	17 (51.5)	8 (61.5)	.816
History of manifest stroke, *n* (%)	6 (18.2)	0 (0)	<.001
History of acute chest syndrome, *n* (%)	4 (12.1)	0 (0)	.223
Cardiopulmonary complications, *n* (%)	10 (30.3)	1 (7.6)	.041
HbS at study (%)	65.9 ± 15.9	49.1 ± 14.8	<.001
HbF at study (%)	10.1 (5-16.2)	13.9 (7.3-18.7)	.376
HbA at study (%)	24.1 ± 10.7	43.3 ± 15.6	<.001
WBC count (×10^9/L)	14.8 ± 4.9	11.6 ± 5.1	.078
Pretransfusion hemoglobin (g/dL)	6.5 ± 0.7	7.4 ± 1.0	.008
Lactate dehydrogenase (IU/L)	647.5 ± 194.5	600.5 ± 180.2	.511
Indirect bilirubin (mg/dL)	1.86 ± 0.73	2.1 ± 0.45	.271
Serum ferritin (μg/L)	3008 (1967-5109)	2880 (1600-4910)	.562
LIC (mg/g liver dry weight)	12.6 (8.7-25.0)	12.3 (6.9-18.5)	.474
Cardiac T2* (ms)	31.7 ± 7.7	29.8 ± 8.4	.561

Abbreviations: BMI, body mass index; BP, blood pressure; Hb: hemoglobin; WBC, white blood cell; LIC, liver iron concentration; IQR, inter-quartile range. Data were expressed as mean ± SD where Student's *t*-test was used for comparisons or as median and IQR using Mann-Whitney test for comparison unless specified as number (percentage) where the chi-square (χ^2) test was used.

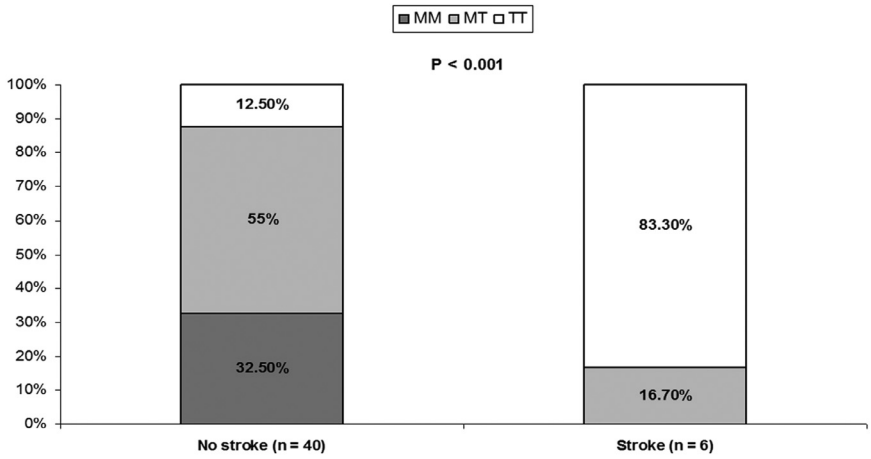

Figure 1. *Genotype frequency of angiotensinogen M235T gene polymorphism in sickle cell disease patients with history of manifest stroke.*

have a greater risk of progression. Regular transfusions to maintain HbS <30% is the preferred first-line treatment for children with first ischemic stroke.[48] But, transfusion alone is inadequate therapy for many patients as progressive cerebral injury occurred in 45% (18/40) of well-transfused children with median follow-up of 5.5 years.[53]

Stroke is a multi-factorial disease and the underlying mechanisms of stroke comprised from both environmental and genetic factors,[54] and some inherited susceptibility genes with single nucleotide polymorphisms may represent their effects in the development of stroke[55] and might help to identify those at increased risk of stroke in SCA patients.[56]

In this study, the frequency of T allele of *AGT* M235T gene polymorphism was significantly associated with higher incidence of stroke as well as higher HbS and lower hemoglobin level compared with those with MM genotype. Of note, T alleles or genotypes were not related to parameters of iron overload in our study implying a mechanism related to HbS polymerization rather than iron overload. Logistic regression analysis indicated that *AGT* M235T gene polymorphism could represent a significant contributing factor for stroke in SCD patients. To our knowledge, this is the first study that assessed *AGT* M235T gene polymorphism as a risk for cerebrovascular stroke in SCD patients.

Fifteen molecular variants have been identified in human *AGT* gene.[28] The *AGT* M235T polymorphism has been associated with increased salt sensitivity,[57] essential hypertension in Caucasian, African-Caribbean, Japanese, and Taiwanese populations[28] and can modify the response of angiotensin converting enzyme (ACE)-inhibitors antihypertensive drugs.[58] It has also been linked to development of cardiovascular diseases,[32] white matter lesions and carotid plaques.[59]

Previous studies suggested that relative hypertension is an increased risk factor for stroke in patients with SCD.[60] An association was found between relative increased blood pressure and the occurrence of occlusive stroke in SCD patients even when their blood pressure is within a normal range compared with normal population.[61]

Over the last decades, many studies have localized their efforts to know the association between stroke and *AGT* M235T gene polymorphism.[34,62] Our results were consistent with other meta-analysis studies that confirmed a significant association between *AGT* M235T gene polymorphism and risk of stroke in Chinese, Asians, and East Asians population.[31,63-65] The TT genotype and T allele are risk factors for ischemic stroke[65] but the relation was not studied in SCD. Tang et al[32] found a significant association between another polymorphism (GT-repeat polymorphism) within the *AGT* gene and the risk of stroke in pediatric patients with SCD (21 stroke and 42 non-stroke subjects). Thus, determination of GT-repeat of *AGT* gene may be a useful genetic marker to assess the risk for stroke of patients with SCD.

Table 3. *Multivariable logistic regression analysis of independent variables related to the occurrence of stroke among patients with sickle cell disease*

		95% confidence interval for OR		
Independent variable	Odds ratio (OR)	Lower	Upper	*P* value
Pretransfusion hemoglobin (g/dL)	1.87	.67	7.15	.356
HbS (%)	2.18	1.23	4.27	.01
M235T genotype	14.05	3.28	28.91	.001
M235T allele	29.42	4.79	41.76	.001

Nakase et al[66] examined major clinical risk factors and the *AGT* gene M235T polymorphism in 147 consecutive stroke Japanese patients and 133 healthy age-matched controls. The authors reported that the *AGT* gene M allele significantly increased the risk of lacunar infarctions in men, independent of hypertension.

In SCA patients, blood viscosity is a risk factor for stroke but the pathogenesis is still unclear. Increased blood viscosity lead to further impair the blood flow, and in SCA patients, blood viscosity is dependent on a group of risk factors including HbS concentration, total hemoglobin concentration, and red blood cell deformability in deoxygenated and oxygenated states.[67,68] We found that HbS was a significant independent variable for the occurrence of stroke in SCD according to logistic regression analysis. However, we could not find any significant association between the occurrence of stroke and the level of pre-existing anemia which may be because all SCD patients whether with or without stroke were transfused at a low pretransfusion hemoglobin concentration around 7 g/dL. A previous Egyptian cross sectional study, on same center, included 205 Egyptians β-TM patients found that patients transfused at low hemoglobin levels. The authors explained this finding by the scarcity of blood available for the patients, which may reflect negative cultural attitudes toward blood donation as well as limited resources and illustrates a significant public health concern for a developing country like Egypt.[69]

Other contributing risk factors for stroke include higher baseline systolic blood pressure[61] but only one patient in our study had blood pressure >95th percentile for age and he did not experienced stroke. We also could not find any relation between smoking and the occurrence of stroke. This could be explained by the young age of the studied group and that only two patients were smokers.

In this study, we found that the T allele of *AGT* M235T gene polymorphism may be considered a genetic modifier for cardiovascular morbidities in Egyptian patients with SCD. Schelleman et al[33] showed that the risk of myocardial infarction was increased in current use of ACE-inhibitors with the MT or TT genotype of *AGT* M235T gene polymorphism compared to ACE-inhibitors with the MM genotype and stated that ACE-inhibitor users with at least one copy of the 235T-allele of the *AGT* gene might have an increased risk of myocardial infarction and stroke. It has been reported that a combination of genotype variants of the RAS genes is a powerful determinant of subclinical progression of coronary artery atherosclerosis in type 1 diabetic patients.[70]

As regards the prospective biological efficacy of *AGT* M235T gene polymorphism, the results from our study suggest that T allele of *AGT* M235T gene polymorphism was associated with higher incidence of stroke and cardiopulmonary complications. This emphasizes the potential importance of RAS, and of this genomic region in particular, for early detection of these morbidities among SCD patients and, in the future, *AGT* M235T gene polymorphism may become an important part of the clinical process of risk identification. It may also be a future therapeutic target for prevention of these SCD-related vascular complications. The mechanism(s) by which this polymorphism affects stroke or cardiovascular morbidities remain to be elucidated.

Limitations of the study include the small number of SCD patients. Since the study was not a multicenter one, we could not include except patients admitted to our hospital as a sample of population in a developing Middle East country with distinctive genetic background and environmental factors. Although our findings were clear and indicative of a significant association between *AGT* M235T gene polymorphism and stroke as well as cardiopulmonary complications in pediatric patients with SCD, they need to be confirmed in larger multicenter studies.

In conclusion, *AGT* M235T gene polymorphism provides further insights on the pathophysiology of cerebrovascular and cardiac morbidities in SCD. *AGT* M235T gene polymorphism may represent a genetic modifier to the occurrence of stroke and cardiopulmonary complications in Egyptian patients with SCD. Thus, it could provide utility for vascular risk assessment. Larger longitudinal studies are necessary to verify the role of *AGT* M235T gene polymorphism in SCD and allow for earlier therapeutic intervention. It would be of interest to investigate the distribution of angiotensinogen M235T polymorphism among Egyptian adults with and without SCD and stroke.

References

1. Bender MA, Hobbs W. Sickle cell disease. In: Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP, eds. GeneReviews, Seattle: University of Washington; 1993-2003 http://www.ncbi.nlm.nih.gov/books/NBK1377/.
2. McCavit TL. Sickle cell disease. Pediaterics Rev 2012;33:195-206.
3. Chang AK, Ginter Summarell CC, Birdie PT, et al. Genetic modifiers of severity in sickle cell disease. Clin Hemorheol Microcirc 2018;68:147-164.
4. Ohene-Frempong K, Weiner SJ, Sleeper LA, et al. Cerebrovascular accidents in sickle cell disease: rates and risk factors. Blood 1998;91:288-294.
5. Jude MA, Aliyu GN, Nalado AM, et al. Stroke prevalence amongst sickle cell disease patients in Nigeria: a multicentre study. Afr Health Sci 2014;14:446-452.
6. Ware RE, Davis BR, Schultz WH, et al. Hydroxycarbamide versus chronic transfusion for maintenance of transcranial doppler flow velocities in children with sickle cell anaemia-TCD With Transfusions Changing to Hydroxyurea (TWiTCH): a multicentre, open-label, phase 3, noninferiority trial. Lancet 2016;387:661-670.
7. Prengler M, Pavlakis SG, Prohovnik I, et al. Sickle cell disease: the neurological complications. Ann Neurol 2002;51:543e52.
8. Ohene-Frempong K, Weiner SJ, Sleeper LA, et al. Cerebrovascular accidents in sickle cell disease rates and risk factors. Blood 1998;91:288-294.

9. Steinberg MH, Sebastiani P. Genetic modifiers of sickle cell disease. Am J Hematol 2012;87:795-803.
10. Driscoll MC, Hurlet A, Styles L, et al. Stroke risk in siblings with sickle cell anemia. Blood 2003;101:2401-2404.
11. Switzer JA, Hess DC, Nichols FT, et al. Pathophysiology and treatment of stroke in sickle-cell disease: present and future. Lancet Neurol 2006;5:501-512.
12. Wang WC. The pathophysiology, prevention, and treatment of stroke in sickle cell disease. Curr Opin Hematol 2007;14:191-197.
13. Steinberg MH. Genetic etiologies for phenotypic diversity in sickle cell anemia. Scientific World J 2009;9:46-67.
14. Thein SL, Menzel S. Discovering the genetics underlying foetal haemoglobin production in adults. Br J Haematol 2009;145:455-467.
15. Fertrin KY, Costa FF. Genomic polymorphisms in sickle cell disease: implications for clinical diversity and treatment. Expert Rev Hematol 2010;3:443-458.
16. Thein SL. Genetic modifiers of sickle cell disease. Hemoglobin 2011;35:589-606.
17. Hsu LL, Miller ST, Wright E, et al. Alpha Thalassemia is associated with decreased risk of abnormal transcranial Doppler ultrasonography in children with sickle cell anemia. J Pediatr Hematol Oncol 2003;25:622-628.
18. Belisario AR, Rodrigues CV, Martins ML, et al. Coinheritance of alpha-thalassemia decreases the risk of cerebrovascular disease in a cohort of children with sickle cell anemia. Hemoglobin 2010;34:516529.
19. Flanagan JM, Frohlich DM, Howard TA, et al. Genetic predictors for stroke in children with sickle cell anemia. Blood 2011;117:6681-6684.
20. Wang WC, Pavlakis SG, Helton KJ, et al. MRI abnormalities of the brain in one-year-old children with sickle cell anemia. Pediatr Blood Cancer 2008;51:643-646.
21. Kwiatkowski JL, Zimmerman RA, Pollock AN, et al. Silent infarcts in young children with sickle cell disease. Br J Haematol 2009;146:300-305.
22. Hoppe C, Klitz W, Cheng S, et al. Gene interactions and stroke risk in children with sickle cell anemia. Blood 2004;103:2391-2396.
23. Paternoster L, Chen W, Sudlow CL. Genetic determinants of white matter hyperintensities on brain scans: a systematic assessment of 19 candidate gene polymorphisms in 46 studies in 19,000 subjects. Stroke 2009;40:2020-2026.
24. Sparks MA, Crowley SD, Gurley SB, et al. Classical renin-angiotensin system in kidney physiology. Compr Physiol 2014;4:1201.. -128.
25. Choi H, Kang JY, Yoon HS, et al. Association of angiotensin-converting enzyme and angiotensinogen gene polymorphisms with preeclampsia. J Korean Med Sci 2004;19:253-257.
26. Wang JG, Staessen JA. Genetic polymorphism in the renin-angiotensin system: relevance for susceptibility to cardiovascular disease. Eur J Pharmacol 2002;410:289-302.
27. Afshariani R, Roozbeh J, Sharifian M, et al. Association between angiotensinogen M235T polymorphism and preeclampsia in Iranian pregnant women. J Fam Reprod Health 2014;8:169-173.
28. Say YH, Ling KH, Duraisamy G, et al. Angiotensinogen M235T gene variants and its association with essential hypertension and plasma renin activity in Malaysian subjects: a case control study. BMC Cardiovasc Disord 2005;5:7.
29. Shamaa MM, Fouad H, Haroun M, Hassanein M, Abdel Hay MA. Association between the angiotensinogen (AGT) gene (M235T) polymorphism and essential hypertension in Egyptian patients. Egypt Heart J 2015;67:1-5.
30. Kooffreh ME, Anumudu CI, Akpan EE, et al. A study of the M235T variant of the angiotensinogen gene and hypertension in a sample population of Calabar and Uyo, Nigeria. Egypt J Med Hum Genet 2013;14:13-19.
31. Bao H, Hao JJ, Yang YM, et al. Angiotensinogen polymorphism and ischemic stroke risk. Int J Clin Exp Med 2015;8:12914-12920.
32. Tang DC, Prauner R, Liu W, et al. Polymorphisms within the angiotensinogen gene (GT-repeat) and the risk of stroke in pediatric patients with sickle cell disease: a case-control study. Am J Hematol 2001;68:164-169.
33. Schelleman H, Klungel OH, Witteman JC, et al. Angiotensinogen M235T polymorphism and the risk of myocardial infarction and stroke among hypertensive patients on ACE-inhibitors or beta-blockers. Eur J Hum Genet 2007;15:478-484.
34. Liang X, Qiu J, Liu X, et al. Polymorphism of angiotensinogen gene M235T in myocardial infarction and brain infarction: a meta-analysis. Gene 2013;529:73-79.
35. Yao JL, Sun SJ, Zhou YF, et al. Association between angiotensinogen M235T polymorphism and hypertrophic cardiomyopathy. Int J Clin Exp Med 2015;8:8767-8775.
36. Sarkar S, Gupta V, Kumar A, et al. M235T polymorphism in the AGT gene and A/G(I8-83) substitution in the REN gene correlate with end-stage renal disease. Nephron 2015;129:104-108.
37. Zhou TB, Yin SS, Qin YH. Association of angiotensinogen M235T gene polymorphism with end-stage renal disease risk: a meta-analysis. Mol Biol Rep 2013;40:765-772.
38. Wethers DL. Sickle cell disease in childhood: Part I. Laboratory diagnosis, pathophysiology and health maintenance. Am Fam Physician 2000;62:1013-1020.
39. Kwiatkowski JL. Hemoglobinopathies. In: Lanzkowsky P, ed. Manual of Pediatric Hematology and Oncology, 5th ed., Elsevier; 2011. p. 221.
40. Ataei N., Hosseini M., Fayaz M., et al. Blood pressure percentiles by age and height for children and adolescents in Tehran, Iran. J Hum Hypertens; 30:268-277.
41. Pashankar FD, Carbonella J, Bazzy-Asaad A, et al. Prevalence and risk factors of elevated pulmonary artery pressures in children with sickle cell disease. Pediatrics 2008;121:777-782.
42. Morris CR, Kim HY, Trachtenberg F, et al. Risk factors and mortality associated with an elevated tricuspid regurgitant jet velocity measured by Doppler-echocardiography in thalassemia: a Thalassemia Clinical Research Network report. Blood 2011;118:3794-3802.
43. Aessopos A, Farmakis D, Deftereos S, et al. Thalassemia heart disease: a comparative evaluation of thalassemia major and thalassemia intermedia. Chest 2005;127:1523-1530.
44. Hankins JS, McCarville MB, Loeffler RB, et al. R2* magnetic resonance imaging of the liver in patients with iron overload. Blood 2009;113:4853-4855.
45. Carpenter JP, He T, Kirk P, Roughton M, et al. On T2* magnetic resonance and cardiac iron. Circulation 2011;123:1519-1528.
46. DeBaun MR, Sarnaik SA, Rodeghier MJ, et al. Associated risk factors for silent cerebral infarcts in sickle cell anemia: low baseline hemoglobin, sex, and relative high systolic blood pressure. Blood 2012;119:3684-3690.
47. Roach ES, Golomb MR, Adams R, et al. Management of stroke in infants and children: a scientific statement from a

Special Writing Group of the American Heart Association Stroke Council and the Council on Cardiovascular Disease in the Young. Stroke 2008;39:2644-2691.
48. Webb J, Kwiatkowski JL. Stroke in patients with sickle cell disease. Expert Rev Hematol 2013;6:301-316.
49. Strouse JJ, Lanzkron S, Urrutia V. The epidemiology, evaluation and treatment of stroke in adults with sickle cell disease. Expert Rev Hematol 2011;4:597-606.
50. Scothorn DJ, Price C, Schwartz D, et al. Risk of recurrent stroke in children with sickle cell disease receiving blood transfusion therapy for at least five years after initial stroke. J Pediatr 2002;140:348-354.
51. Kwiatkowski JL, Yim E, Miller S, et al. Effect of transfusion therapy on transcranial Doppler ultrasonography velocities in children with sickle cell disease. Pediatr Blood Cancer 2011;56:777-782.
52. Bishop S, Matheus MG, Abboud MR, et al. Effect of chronic transfusion therapy on progression of neurovascular pathology in pediatric patients with sickle cell anemia. Blood Cells Mol Dis 2011;47:125-128.
53. Hulbert ML, Mckinstry RC, Lacey JL, et al. Silent cerebral infarcts occur despite regular blood transfusion therapy after first strokes in children with sickle cell disease. Blood 2011;117:772-779.
54. Goldstein LB, Adams R, Alberts MJ, et al. Primary prevention of ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council: cosponsored by the Atherosclerotic Peripheral Vascular Disease Interdisciplinary Working Group; Cardiovascular Nursing Council; Clinical Cardiology Council; Nutrition, Physical Activity, and Metabolism Council; and the Quality of Care and Outcomes Research Interdisciplinary Working Group: the American Academy of Neurology affirms the value of this guideline. Stroke 2006;37:1583-1633.
55. Hassan A, Markus HS. Genetics and ischaemic stroke. Brain 2000;123:1784-1812.
56. Zaheer Z, Robinson T, Mistri AK. Thrombolysis in acute ischaemic stroke: an update. Ther Adv Chronic Dis 2011;2:119-131.
57. Katsuya T, Ishikawa K, Sugimoto K, et al. Salt sensitivity of Japanese from the viewpoint of gene polymorphism. Hypertens Res 2003;26:521-525.
58. Bis JC, Smith NL, Psaty BM, et al. Angiotensinogen Met235Thr polymorphism, angiotensin-converting enzyme inhibitor therapy, and the risk of nonfatal stroke or myocardial infarction in hypertensive patients. Am J Hypertens 2003;16:1011-1017.
59. Van Rijn MJ, Schut AF, Aulchenko YS, et al. Heritability of blood pressure traits and the genetic contribution to blood pressure variance explained by four blood pressure-related genes. J. Hypertens 2007;25:565-570.
60. Rodgers GP, Walker EC, Podgor MJ. Is relative hypertension a risk factor for vaso-occlussive complications in sickle cell disease. Am J Med Sci 1993;305:150-156.
61. Pegelow CH, Colangelo L, Steinberg M, et al. Natural history of blood pressure in sickle cell disease: risks for stroke and death associated with relative hypertension in sickle cell anemia. Am J Med 1997;102:171-177.
62. Brand E, Chatelain N, Paillard F, et al. Detection of putative functional angiotensinogen (AGT) gene variants controlling plasma AGT levels by combined segregation-linkage analysis. Eur J Hum Genet 2002;10:715-723.
63. Liang B, Qin L, Wei H, et al. AGT M235T polymorphisms and ischemic stroke risk: a meta-analysis. J Neurol Sci 2013;331:118-125.
64. Gao T, Huang L, Fu Q, et al. Association of polymorphisms in the AGT gene (M235T, T174M) with ischemic stroke in the Chinese population. J Renin Angiotensin Aldosterone Syst 2015;16:681-686.
65. Wang S, Zeng R, Lei L, et al. Angiotensinogen gene polymorphism and ischemic stroke in East Asians: a meta-analysis. Neural Regen Res 2013;8:1228-1235.
66. Nakase T, Mizuno T, Harada S, et al. Angiotensinogen gene polymorphism as a risk factor for ischemic stroke. J Clin Neurosci 2007;14:943-947.
67. Itoh T, Chien S, Usami S. Effects of hemoglobin concentration on deformability of individual sickle cells after deoxygenation. Blood 1995;15:2245-2253.
68. Silva CM, Giovani P, Viana MB. High reticulocyte count is an independent risk factor for cerebrovascular disease in children with sickle cell anemia. Pediatric Blood Cancer 2011;56:116-121.
69. Adly AA, Ebeid FS. Cultural preferences and limited public resources influence the spectrum of thalassemia in Egypt. J Pediatr Hematol Oncol 2015;37:281-284.
70. Kretowski A, McFann K, Hokanson JE, et al. Polymorphisms of the renin-angiotensin system genes predict progression of subclinical coronary atherosclerosis. Diabetes 2007;56:863-871.