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Association of Thrombomodulin C1418T Polymorphism with Vaso-occlusive Crisis in Patients with Sickle Cell Anaemia

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Abstract

Background: Sickle cell anemia (SCA) is a genetic disorder caused by homozygosity for single β–globin gene mutation, in which glutamic acid has been substitute for valine at sixth codon of β-globin chain. Severity of sickle cell anemia results from the vaso-occlusive crisis, or sickle cell crisis, which is a common painful complication of sickle cell disease in adolescents and adults. Several published studies have shown that the Ala455Val thrombomodulin polymorphism is associated with ischemic heart disease and atherosclerotic diseases, but none has examined the association with vaso-occlusion. Data from sickle patients with vaso-occlusion were used to determine the association between the Ala455Val thrombomodulin polymorphism and the occurrence of vaso-occlusion in children with sickle cell anemia.

Objective: To determine the association of thrombomodulin C1418T polymorphism with vaso-occlusive crisis in patients with sickle cell anaemia.

Materials and methods: This was a hospital-based, case-control study conducted at Jafar Ibn Ouf Specialized Hospital for children (Khartoum, Sudan), during the period from August to December, 2018. Blood specimens were collected from 40 children with sickle cell anemia (test group) and 40 apparently healthy volunteers (control group). Genomic DNA was extracted from the peripheral leucocytes and used for detection of thrombomodulin C1418T polymorphism. Patients' data was collected from medical files and analyzed by the Statistical Package for Social Sciences.

Results: The age range of the test group patients was 1-18 years (mean 8.5 ± 5.03 SD); and 22 of them (55.0%) were males and 18 (45.0%) were females. The age range of the control group participants was also 1-18 years (mean 7.7 ± 5.11 SD); and 16 (40%) of them were males and 24 (60%) were females. Thrombomodulin C1418T polymorphism with vaso-occlusive crisis in the test group patients was CC 25 (62.5%), CT 14 (35%), and TT 1 (2.5%); and that in the control group patients was CC 18 (45.0%), CT 20 (50%) and TT 2 (5.0%). This difference was statistically insignificant (p = 0.282).

Conclusion: . The thrombomodulin gene had a role in production of the vaso-occlusive crises in sickle cell anemia patients. Thrombomodulin genotype CC had a higher frequency rate as compared with most thrombomodulin genotype in sickle cell anemia test group patients. Thrombomodulin genotype TT had a lower frequency rate as compared with most thrombomodulin genotype in sickle cell anemia test group patients. Thrombomodulin genotype

CT had a higher frequency rate as compared most thrombomodulin genotype in control group participants. No correlation was found between thrombomodulin genotype according to gender and age incidence.

Keywords: Sickle cell anaemia, Thrombomodulin, Polymorphism, Vaso-occlusive crisis.

Introduction

Sickle cell anemia (SCA) is an inherited autosomal recessive disease characterized by the presence of the homozygous hemoglobin S (Hb S). It is caused by a single nucleotide mutation that substitutes glutamic acid for valine in the sixth position of the globin gene. During hypoxic conditions, the red blood cell becomes sickled and the resulting change in structure restricts circulation causing obstruction of the blood flow within the capillaries and early destruction of the cell. Sickle cell disease (SCD) is reported to be associated with a very high rate of childhood mortality[1].

The most common complication of SCD is an acute episode of severe pain referred to as an acute vaso-occlusive crisis (VOC). The sickled erythrocytes block the flow of blood through the small blood vessels (capillaries) resulting in ischemia. Sudden episodes of pain throughout the body are a common symptom of SCD. This sudden pain can range from mild to a very severe form and usually lasts from hours to a few days. VOCs and their accompanying pain most commonly occur in the extremities, chest, and back. When they occur in other sites, they can be confused with, or can be the prodromal stage of other acute complications (e.g. head (stroke), flank (papillary necrosis), and abdomen (hepatic or splenic sequestration, constipation from opioid toxicity, or another hepatobiliary complication). Patients with more than three hospitalizations for a VOC in a year are considered to be at an increased risk of early death[2].

When sickle cells block the small blood vessels at hands or feet, pain and swelling along with fever may occur. The first VOC may appear as early as 6 months of age, often presenting as dactylitis, but thereafter VOCs occur with variable frequency. This may be the first sign of sickle cell anemia in infants. Other common acute complications of SCD include fever related to infection, acute renal failure, hepatobiliary complications, acute anemia, splenic sequestration, acute chest syndrome (ACS), and acute stroke. Thrombomodulin (TM) is a critical cofactor in the initiation of the protein C (PC) anticoagulant pathway . It is a glycosylated type I transmembrane molecule of 557 amino acids and does not possess intrinsic enzymatic activity. About half the extracellular domain consists of an N-terminal globular domain with weak sequence similarity to C-type animal lectins[3].

The TM–thrombin complex is a potent activator of PC that enhances thrombin dependent PC activation by more than two orders of magnitude. Due to the abundance of TM in the microvasculature, the vast majority of thrombin generated under ambient conditions is sequestered by TM. Constitutive inhibition of the procoagulant function of thrombin and tonic activated protein C (APC) formation provide an essential anticoagulant mechanism that prevents the amplification of thrombin generation, via proteolysis of activated coagulation factors Va and VIIIa by APC. In addition to the cofactor function of TM in the activation of PC, the TM thrombin complex also activates latent plasma carboxypeptidase B enzymes e.g. thrombin activatable fibrinolytic inhibitor (TAFI). Single nucleotide polymorphism at position C.1418

C >T of TM gene causes transition of cytosine (C) to thymidine (T) that results in substitution alanine (A) to valine (V) at protein position 455 (ala 455 Val) of the TM gene. The link between venous thrombosis and the Ala455Val dimorphism within the sixth EGF domain has been evaluated in several studies with contradictory results[4].
This study aimed to investigate the association between TM C1418T polymorphism and incidence of vaso-occlusive crisis in patients with sickle cell anaemia.

Materials and methods

This was a case-control study, conducted during the period from August to December, 2018. The study was approved by the Scientific Research Committee of Al Neelain University, Khartoum (Sudan), and the Ethical Board of the Ministry of Health (Sudan). Data confidentially was maintained, and the information collected from all specimens had not been used for any purpose other than this study. Permission to collect the specimens was granted from the director of Jafar Ibn Ouf Specialized Hospital for Children, Khartoum (Sudan). Informed consent was obtained from patients' parents before samples collection.40 children with sickle cell anemia and had vaso-occlusive crises (test group), attending Jafar Ibn Ouf Specialized Hospital for Children, Khartoum (Sudan). 22 (55.0%) of the patients investigated were males and 18 (45.0%) were females (mean age was 7.7 years). Another 40 age-and gender-matched, apparently healthy participants were also enrolled as a control group. Patients with known genetic or acquired cause of hypercoagulability were excluded from the study.
Three milliliter (ml) of venous blood were collected in EDTA containers and genomic DNA was extracted by the salting-out method. Allele specific-polymerase chain reaction (AS-PCR) was used for analysis of TM C1418T polymorphism.
A common forward primer (5′-TCAGAGC CAACTGCGAGTACC-3′) and two allele-specific reverse primers, C Allele (5′-ACAGTCGGTGCCAATGTGGCGTG-3′) and T Allele (5′-ACAGTCGGTGCCAAT GTGGCGTA-3′) were used.
Product size was 332 (bp) for all primers. PCR mixture of 20µl was prepared for each sample in sterile Eppendrof tube, included the C allele, was D.W 13 µl, forward primer 0.5 µl, reverse primers C allele 0.5 µl, Template DNA 3 µl, and Master mix 3 µl. The content of T allele was D.W 13 µl, forward primer 0.5 µl, reverse primers T allele 0.5 µl, Template DNA 3 µl and Master mix 3 µl (Maxime PCR Premix kit, i-Tag ^{TM}).
The optimized cycling conditions consisted of initial denaturation at 95°C for 5 minutes, followed by 35 cycle each consisting of denaturation at 95^{o} C for 20 seconds, annealing at 58^{o} C for 20 seconds and extension at 72^{o} C for 30 seconds, followed by final extension at 72^{o} C for 5 minutes. Five µl of the PCR product (ready to load) was electrophoresed on 2% agarose gel containing ethidium bromide; 100 bp DNA ladder was applied with each batch of samples. Finally, PCR product was demonstrated by gel documentation system (Syngene).
Statistical analysis: Data was collected from patient's files. Frequencies, mean values, standard deviations (SD) and ranges were used as descriptive statistics. The positivity was tested by means of the Mantel-Haenszel chi-square test for linear association. The statistical analysis was performed by running the SPSS/PC+ statistical package version 20, on a personal computer.
A two-tailed P of 0.05 was chosen as the cut-off for detecting statistically significant values.

Results

Using a specific primer, the TM C1418T polymorphism was obtained from ASP-PCR. There were 25 (62.5%) patients with the CC genotype, 14 (35.0%) patients with the CT genotype, and 1 (2.5%) patient with the TT genotype. In the control group there were 18 (45.0%), 20 (50.0%) and 2 (5.0%) for the CC, CT and TT genotypes respectively.
As shown in Table (1), the frequency rate of thrombomodulin C1418T polymorphism genotype CC allele was found higher (62.5%) in the test group patients than CC allele of control group volunteers (45.0%). CT polymorphism was found lower (35%) in test group than control group (50.0%) participants. TT was (2.5%) in test group patients and was (5.0%) in control group participants, showing a too close result. Additionally, there were no statistically significant differences in the genotypes of test group patients and those of control group participants ($p = 0.419$).

Table (1): Distribution of C1418T polymorphism genotypic variants in test group patients and control group participants

Parameter	Test group patients	Control group participants	p - value
CC	25 (62.5%)	18 (45.0%)	0.419
CT	14 (35.0%)	20 (50.0%)	
TT	1 (2.5%)	2 (5.0%)	

The CC genotype was the most frequent in both males and females followed by the genotypes CT and TT respectively. No statistically significant difference ($p = 0.335$) was found in genotypes distribution regarding gender (Table 2).

Table (2): Distribution of C1418T polymorphism genotypic variants according to gender

Parameter	Males	Females	p - value
CC	13 (59.1%)	22 (66.7%)	0.335
CT	9 (40.9%)	5 (27.8%)	
TT	0 (0%)	1 (5.6%)	

In the age range 1-9 years, C1418T polymorphism was CC 15 (65%) and CT 8 (34.8%), while in the age range 10-18 years, it was TT 0 (0.0%). There was no statistically significant association between the thrombomodulin C1418T polymorphism and the age incidence ($p = 0.492$).
The thrombomodulin C1418T polymorphism in patients investigated was: CC 10 (58.8%), CT 6 (35.3%), and TT 1 (5.9%). There was no statistically significant difference between the thrombomodulin C1418T polymorphism and patients ($p = 0.282$).
Thrombomodulin C1418T polymorphism associated with vaso-occlusive crisis in test group patients was: CC 25 (62.2%), CT 14 (35%); and control group participants was: TT 1 (2.5%), CC 18 (45.0%), CT 20 (50.0%) and TT 2 (5.0%).

Discussion

The present study studied the association of thrombomodulin C1418T polymorphism with vaso-occlusive crisis in patients with sickle cell anaemia to compare with control healthy persons. The result showed that the frequency rate of thrombomodulin polymorphic genotype CC was higher (62.5%) in test group patients than that in control group volunteers (45.0%). Also, the CT and TT genotypes were found higher in the control group volunteers than test group patients (50% vs 35% and 5% vs 2.5% respectively). The prevalence rate of the CC genotype among the control group volunteers was similar to that of test group patients with vaso-occlusive crises. These findings indicated a negative association between thrombomodulin C1418T polymorphism and vaso-occlusive crises among the 1-18 years patients investigated.

The findings of this study was in agreement with the findings of a study conducted by Cole and his colleagues[5-] (37) who reported that prevalence rate of CC genotype among test group and control group cases was 81% among black Americans and 68% among the whites. The black Americans were found significantly more likely to have the CC genotype than the CT and TT genotypes combined (38.8% vs. 24.1%, $p < 0.05$). However, the findings of this study disagreed with Cole and his colleagues[5] study in the association of thrombomodulin genotype with vaso-occlusive crises who found a positive association between the thrombomodulin polymorphism and vaso-occlusion.

Recommendations: Further large sample size studies at the molecular level are needed to support the findings of the present studies, and to correlate the gene polymorphism among sickle cell patients and to establish the treatment of the clinical crisis episodes.

Conclusion: The thrombomodulin gene had a role in production of the vaso-occlusive crises in sickle cell anemia patients. Thrombomodulin genotype CC had a higher frequency rate as compared with most thrombomodulin genotype in sickle cell anemia test group patients. Thrombomodulin genotype TT had a lower frequency rate as compared with most thrombomodulin genotype in sickle cell anemia test group patients. Thrombomodulin genotype CT had a higher frequency rate as compared most thrombomodulin genotype in control group participants. No correlation was found between thrombomodulin genotype according to gender and age incidence.

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