International Journal of Multidisciplinary and Current Research ISSN: 2321-3124
Research Article Available at: http://ijmcr.com

Association of genotypic variants of vascular cell adhesion molecule -1 with the risk of stroke in Sudanese sickle cell anemia patients

Tyseer Alabid[1], Anwaar A.Y.Kordofani[2], Bakhieta Atalla[3], Hisham N. Altayb[4], Hosham Abdalrahman[5], Afra Abd Ehamid Fadla[6], Marwa Mohamed Osman[6], Bahaeldin K. Elamin[7,8]

[1]Department of Haematology , Faculty of medical laboratory Sciences, University of Khartoum ,Khartoum, Sudan.
[2]Department of Pathology , Faculty of Medicicne , University of Khartoum , Khartoum,Sudan.
[3]Department of Pediatrics, University of Bahr El Gazal, Khartoum,Sudan
[4]Department of Microbiology, Faculty of Medical Laboratory Sciences, Sudan University of Science and Technology, Khartoum, Sudan
[5]Department of Molecular biology , Faculty of medical laboratory Sciences, University of Khartoum , Khartoum, Sudan
[6] Department of biotechnology, Africa city of technology, Sudan
[7]Department of Microbiology, Faculty of medical laboratory Sciences, University of Khartoum , Khartoum, Sudan
[8]Department of Microbiology and Parasitology, College of Medicine , University of Bisha ,Bish, Saudi Arabia

Accepted 01 June 2016, Available online 11 June 2016, **Vol.4 (May/June 2016 issue)**

Abstract

Background: *Sickle cell anemia (SCA) is a monogenic disorder caused by a homozygous mutation in the β-globin gene resulting in early death and morbidity. Stroke is one of its most devastating complications. Vascular cell adhesion molecule 1 gene postulated to play a critical role in the pathogenesis of SS disease. Previous evidence has demonstrated that the nonsynonymous SNP, VCAM11238G > C(rs3783613), may be associated with protection from stroke while1594T > C (rs1041163) SNP had an association with stroke.* ***Methodology:*** *This was a cross-sectional, randomized study that included 131 patients diagnosed with (SCA), among them 40 subjects with history of clinical stroke and 91 subjects with other complications of (SCA). Genotyping of the VCAM1 SNPs was carried out using PCR-RFLP technique and confirmed by sequencing a subset of samples. The results were analyzed using bioinformatics tools. The aim of the present study was to detect the presence of VCAM1 SNPs(rs3783613) and (rs1041163) among Sudanese patients with(SCA) with and without ischemic stroke respectively to determine whether it has an influence on the risk of stroke.* ***Results:*** *Molecular analysis revealed presence of VCAM1 T1594 C mutant genotype among the stroke study group in only 21/40 (52.5%). We also found almost complete absence of VCAM1 G1238C polymorphism among the non-stroke patients.* ***Conclusion:*** *No association between VCAM1 1594Cvariant and stroke in our population of patients. No protective correlation between the VCAM11238C variant and the non-stroke patients of (SCA) patients included in this study.*

Keywords*: ischemic stroke, VCAM1, rs3783613, rs1041163, sickle cell anemia.*

1. Introduction

Sickle cell anemia (SCA) is a monogenic disorder caused by a homozygous mutation in the β-globin gene (HBB:c.20ANT, p.E6V), resulting in early death and morbidity [1].Stroke is a major complication of sickle cell (SS) disease and is associated with significant morbidity and mortality. It is estimated that the lifetime risk for stroke is between 8% and 10%.[2], [3] Associated risk factors include peripheral leukocytosis, [2],[3]the rate of acute chest syndrome (ACS) episodes,[2] relative hypertension,[4]abnormal cerebral blood flow detected by transcranial doppler ultrasonography, and selected genetic variants.[5], [6]**.**Candidate genes, stroke susceptibility alleles and their association with stroke pathogenesis have been intensely studied in the last few years [7]-[9]. Immunity and inflammation are key elements of the pathobiology of ischemic stroke [10], [11].The aim of this study was to investigate the association between certain VCAM1 gene polymorphisms and the risk of and protection from ischemic stroke in Sudanese patients with SCA. Among the genes associated with stroke in sickle cell anaemia, vascular adhesion molecule-1(VCAM1) gene (ID: 7412 / MIM: 192225) postulated to play a critical role in the pathogenesis of SS disease. VCAM-1 is a cell surface sialo glycoprotein immunoglobulin superfamily member highly expressed on endothelial cells following cytokine stimulation with interleukin 1 alpha (IL-1alpha), tumor necrosis factor alpha (TNF-alpha), and inter interleukin 4 (IL-4) [12].Elevated levels of soluble VCAM-1 have been detected in the plasma of patients with SS disease at baseline and during episodes of acute chest syndrome(ACS)[13],[1]. Genetic polymorphisms of VCAM-

1 have been implicated in susceptibility to a number of degenerative and inflammatory diseases [14]-[18]. Previous evidence has demonstrated that the (VCAM-1) gene coding single-nucleotide polymorphism (SNP)1238G >C (rs3783613) may be protective against stroke in SCA and 1594T > C (rs1041163), an intronic SNP located in the promoter of VCAM1gene, had an association with stroke[14],[19]. Also Dworkis et al examined plasma biomarkers of endothelial cell activation for associations with SCA severity in 24 adults (12 mild, 12 severe). (VCAM-1) was significantly higher in subjects with severe SCA. Therefore, Of the 47 SNPs they examined only one SNP, 1594T > C (rs1041163), was significantly associated with SCA severity.[20] However, a recent study found no association between VCAM1 1238G>C and stroke.[21] This finding was supported with another recent study which reported that most of the genetic variants found to be in Hardy-Weinberg equilibrium were not associated to stroke risk or prevention. They reported that the frequency of1594T > C (rs1041163) SNP was as follows: 75% of the obtained alleles were contained the T nucleotide and the remaining 25% contained the C nucleotide, showing no distribution differences among their stroke and non- stroke groups and for the only SNP in the VCAM-1gene they studied, vcam1 1238G>C(rs3783613), 81.8% of the alleles obtained possessed the ancestral nucleotide G and the remaining 18.2% contained the variant C allele.[22] They concluded that these VCAM-1SNPs, both in the promoter and in the gene, which have been implicated as risk modifier variants in previous studies had no association with stroke in (SS) patients.[22] In Sudan no such studies have been carried out. The aim of our study was to investigate the association between VCAM1 T-1594 C gene polymorphism and the risk of ischemic stroke in a Sudanese population group of SCA and also to examine the role of VCAM1G -1238 C gene polymorphism in the protection from stroke in such patients.

Methods

This was a cross-sectional, randomized study involved 131 patients diagnosed with sickle cell anaemia; ages ranged from 6 months to 23 years, 40 subjects among them with history of clinical stroke and 91 without stroke. Seven stroke patients were in their 2nd attack of stroke and one of them was in his 3red one. The study was approved by the Ethics Committee of the Medical Laboratory Sciences (Khartoum University), and all participants were provided a written informed consent form. Diagnosis of stroke was confirmed following neurological assessment and CT scan evaluation in all cases by clinics physician. A sample of 5 ml EDTA venous blood was collected from all patients.

Genotyping

DNA was extracted from 300 µl peripheral blood samples using (QIAamp® DNA Mini Kit using DNA Purification from Blood -Spin Protocol).The VCAM1 T 1594 C and The VCAM1 G1238C polymorphism were detected using a PCR-RFLP method among the stroke and non-stroke patients respectively .Our designed PCR primers (IDT INTEGRATED AND TEQUNELOGY , California) were described as follows: Forward primer 5`-AACACTGTAAAGGACCTCTGG -3’ Reverse primer5’-CTAGGGTGTGGGGATATATAGGT - 3’for theVCAM1 T 1594 C and Forward primer 5`-GGATTTGCAGCCCATCTCAGT-3’ Reverse primer5’- GTA CTC TGC CTT TGT TTG GGT TCG - 3’ for the VCAM1 G 1238 C polymorphism; For the specific genetic analysis, a total amount of 100 ng of genomic DNA was amplified in a total volume of 25 µl reaction mixture (Thermo Fischer Scientific Inc., MA, USA) containing reaction buffer of 1.5 nM MgCl2, 20pmol of each primer, 200 µm of each dNTPs and 0.5 units of Taq polymerase. PCR was carried out using a commercial thermal cycler (Eppendorf Master cycler Thermal Cycler). The amplification steps consisted of an initial 12 minutes denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 seconds each, primer annealing at 60 °C for 30 seconds, primer elongation at 72 °C for 1 minute and a 5 minutes final elongation at 72 °C. The PCR amplification products were digested overnight with 5 units of ALWI enzyme (Fermentas Life Sciences, Vilnius. Lithuania) for VCAM1 T 1594 C and Cac8I for VCAM1 G 1238 C polymorphism; PCR products were treated by restriction enzymes in total volumes of 10 µl according to specific protocols for both SNPs. PCR products were then analyzed by electrophoresis in a 2% agarose gel for the permissive SNP and 1.5% agarose gel for the protective SNP in a TBE 1 X, containing 0.5 µg/ml ethidium bromide at 120V for 45 min for the permissive SNP and 80V for 30 min for the protective SNP.DNA marker from INTRON Biotechnology 50 bp was used. Bands were visualized under U.V transilluminator (Uvite_ UK). The resulted fragments were then separated on a 3 % agarose gel (Caisson, USA) and transilluminated with UV light. Electrophoretic analysis defines 2 distinct banding patterns, each corresponding for 2 possible genotypes. Twenty non-stroke patient's samples did not run through RFLP-PCR due to study limitation so they were sent for sequencing.

Cycle sequencing

Samples were confirmed and completed the genotyping results for RFLP-PCR by sequencing a subset of samples (no=20\40) 50 % of stroke patients and (no=20\91) 22% of non-stroke patients. Standard sequencing was performed for both strands of VCAM1 gene by Macrogen Company (Seoul, Korea). Chromatograms were showed by GATCViewer and FinchTV programs. Sequences were submitted and given accession numbers from GenBank at NCBI database. (Appendix)

Statistical analysis

For statistical analysis we used SPSS 20.0 for Windows. (SPSS, Inc., Chicago, IL). P value and Odds ratio (OR) assessment with 95% confidence limits were calculated by logistic regression.

Bioinformatics analysis

The nucleotides sequences of the two VCAM1 SNPs achieved were searched for sequence similarity using nucleotide BLAST. [23] (http: //blast.nlm.nih.gov/ Blast.cgi). Highly similar sequences were retrieved from NCBI and healthy controls then subjected to multiple sequence alignment using the BioEdit software. We obtained information regarding VCAM1 SNPs, from National Center for Biological Information (NCBI) SNPs database (http:// www.ncbi.nlm.nih.gov/snp/). We analyzed ournsSNP (1238G > C rs3783613) using computational soft wares. For Predicting damaging amino acid substitutions, we used SIFT software (http://sift.jcvi.org/) and for Prediction of functional modification we used Polyphen-2 software (PolyPhen-2, v.2.2.2;http://genetics. bwh.harvard.edu/ pph). The FASTA format of the protein was obtained from Uniprot at Expassy database (http://www.uniprot.org) and submitted in to two servers: I- mutant (http://genetics.bwh.harvard.edu/pph2/index.shtml), and PHD-snp (http://snps.biofold.org/phd-snp/phd-snp.html) tools to further confirmation of the findings of SIFT and Polyphen2. Chimera software (/http:// www.cgl.ucsf.edu/ chimera) was used to generate the mutated 3D (three-dimensional) structure of the nsSNP protein.

Results

Genotyping results

Analysis of the 40 patients who developed stroke for VCAM1 gene polymorphism (T 1594 C) showed that 21 (52.5%) had the TC genotype (positive + polymorphism) while 19 (47.5%) had the TT genotype (negative - polymorphism) by RFLP-PCR (Table 1) and this was confirmed by further sequencing. Among the patients males were more susceptible to have the TC genotype (OR =2.383) than females. Comparative analysis between the stroke patients whose ages were less and more than 10 years revealed a statistically significant difference between them (P value = 0.012), with the risk of having the TC polymorphism being higher in the younger patients (OR = 5.486) (Table 1). Most of the stroke patients had no history of previous one (no=31/40). While 7 patients developed two strokes and only one patient had 3 attacks of stroke and he had the TC genotype (Table 2). Sequencing results of non-stroke patients who did not run RFLP-PCR bands were all negative except for two patients who showed the mutant (VCAM-1) 1238G >C polymorphism.

PCR

The PCR results showed bands typical in size to VCAM1 T1594CSNP (122bp) in 40 stroke patients and bands typical in size to VCAM1 G1238CSNP (825bp) in 91non-stroke patients (Figure 1).

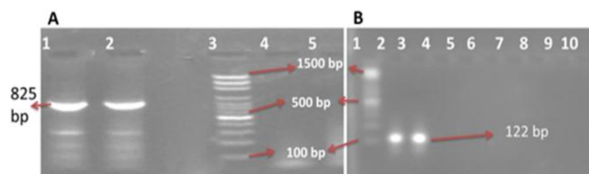

Fig.1. PCR amplification of VCAM 1 gene SNPs in 1.5 % agarose gel electrophoresis. A. Lane 1, 2 non – stroke patients showing typical band size of (825bp) corresponding to the VCAM1 G1238C, with lane3, DNA ladder: MW 100-1500bp fragments. B. Lane 1, DNA ladder: MW 100-1500bp fragments. Lane 2,3 stroke patients showing typical band size of (122bp) corresponding to the molecular size of VCAM1 T1594C SNP

PCR-RFLP

The PCR results showed bands typical in size to VCAM1 T1594CSNP (65bp, 57bp and 122bp) for the stroke patients after digestion by ALWI enzyme and bands typical in size to VCAM1 G1238C SNP (825bp) for the non-stroke patients after digestion by Cac8I enzyme, (Figure 2) and (Figure 3) respectively.

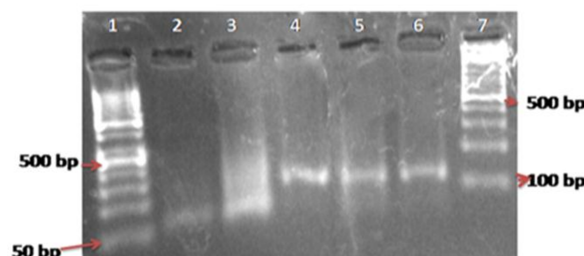

Fig.2. PCR digestion of VCAM1 T1594C SNP in 2 % agarose gel electrophoresis. lane 1: DNA ladder: MW 50-1000bp fragments, lane 7: DNA ladder: MW 100-1500bp fragments. Lane 2,3 were stroke patients showing typical band size of (65, 57 bp) corresponding to the molecular size of VCAM1 T1594C SNP after digestion with ALWI enzyme. Lane 4, 5,6 were stroke patients showing typical band size of (122 bp) corresponding to the molecular size of the undigested VCAM1 T1594C SNP after digestion with ALWI enzyme

Fig.3. PCR digestion of the VCAM1 G1238C SNP in 1.5 % agarose gel electrophoresis. Lane 1 DNA ladder: MW 100-1500bp fragments. Lane 2-11, were non-stroke patients showing typical band size of (825 bp) corresponding to the molecular size of the undigested VCAM1 G1238C SNP after digestion with Cac8I enzyme

Table (1): Distribution of VCAM1 gene polymorphism (T 1594 C) among stroke patients

	(TC SNP) (+) polymorphism (n=21)	(TT SNP) (-) polymorphism (n=19)	P-value	Odds ratio	95% CI
Gender					
Male	11(27.5%)	6(15.0%)	0.184	2.383	**0.655- 8.675**
Female	10(25.0%)	13(32.5%)			
Age					
<10 years	16(40.0%)	7(17.5%)	0.012	5.486	**1.394- 21.591**
>10 years	5(12.5%)	12(30.0%)			

Table (2): Frequency of stroke among study population

(TC SNP) (+) polymorphism	Male	Female	(TT SNP) (-) polymorphism	Male	Female
One stroke	8(38.1%)	8(38.1%)	One stroke	5(26.3%)	11(57.9%)
Two strokes	2(9.5%)	2(9.5%)	Two strokes	1(5.3%)	2(10.5%)
Three strokes	1(4.8%)	0	Three strokes	0	0
Total	11(52.4%)	10(47.6%)	Total	6(31.6%)	13(68.4%)

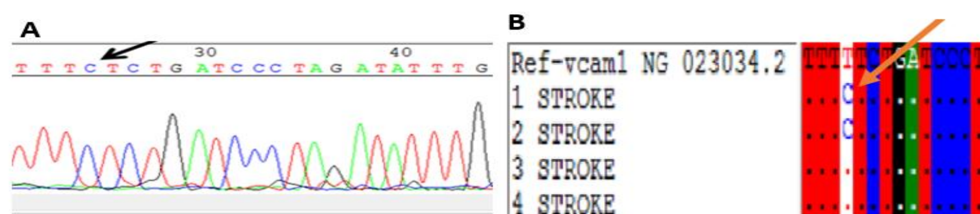

Fig.4. A.T > C substitution sequence chromatogram in stroke patient, shown by GATCViewer program. B. BioEdit multiple sequence alignment showed: first the gene sequence from genebank (Refseq. VCAM1 NG_023034.2) comparing to: (1,2) stroke patients showing VCAM1 1594C variant (mutant type) and (3,4) stroke patients showing VCAM1 1594T variant (wild type)

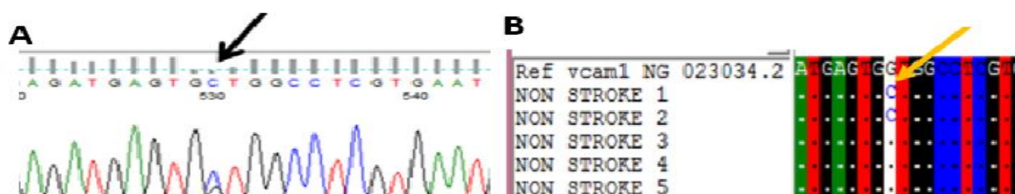

Fig.5. A. G > C substitution sequence chromatogram in non-stroke patients, was shown by FinchTV program. B. BioEdit multiple sequence alignment showed: First the gene sequence from gene bank (Refseq. VCAM1 NG_023034.2) comparing to: non - stroke patients (1,2) showing VCAM1 1238C variant (mutant type) and non-stroke patients (3,4,5) showing VCAM1 1238G variant (wild type)

Chromatograms and Multiple sequence alignments

Chromatograms sent by Macrogene showed substitution from T > C in stroke patient (VCAM1 T1594C SNP), Figure 4(A) in the position 3529 of the gene sequence from genebank (Refseq. VCAM1 NG_023034.2), and substitution from G> C among non-stroke patients (VCAM1 G1238C SNP) in the position 16496 of the gene sequence from genebank (Refseq. VCAM1 NG_023034.2, Figure 5 (A). Alignments by BioEdit software version 7.0.9.0 were carried out among the nucleotides of the VCAM1gene sequences in stroke patients, non-stroke patients and gene sequence from gene bank (Refseq. VCAM1 NG_023034.2). It confirmed the substitution among the stroke and non-stroke patients Figure 4(B), Figure 5 (B) respectively.

Bioinformatics results for VCAM1 G1238C nonsynonymous SNP (rs3783613)

Regarding the SIFT software we found that the G1238C nsSNP presence is predicted to be tolerated unlike the outcomes of the Polyphen 2 software which predicted that the presence of the SNP will be probably damaging to the protein structure. I mutant and PHD –snp servers also were different in their predictions. I mutant confirmed the finding of SIFT software by predicting that the SNP will decrease the protein stability while the PHD-snp predicted the SNP to be neutral polymorphism with no effect on the protein. Table (3) VCAM1 G1238C nonsynonymous SNP (rs3783613) mutation resulted in change of Glutamic acid amino acid into Alanine amino acid at position 413 (G413A).

Table (3): VCAM1 G1238C nonsynonymous SNP (rs3783613) predicted with SIFT, Polyphen, I mutant and PHD-snp **programs.**

SNP	PROTEIN ID	AMINO ACID CHANGE	SIFT PREDICTION	SIFT SCORE	polyphen predicted	polyphen score	I mutant			PHD -snp	
							SVM2 Prediction Effect	RI	DDG Value Prediction	Prediction	RI
Rs3783613	ENSP00000294728	G413A	TOLERATED	0.271	probably damaging	0.999	Decrease	2	-0.84	Neutral	**6**

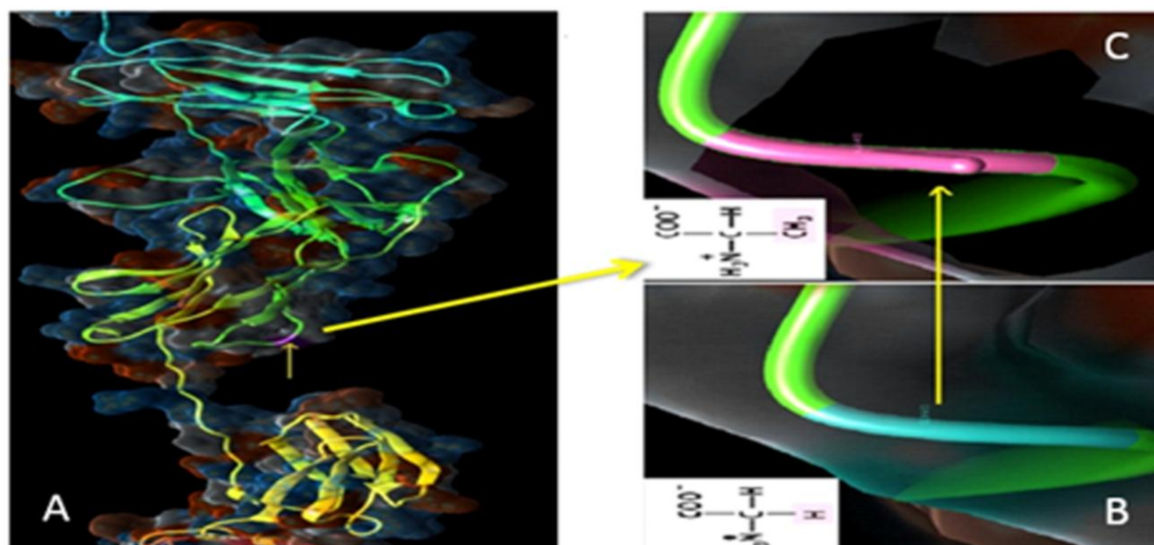

Fig.6.SNP ID:rs3783613, A. represents the vcam1 protein with the arrow pointing to the 413 amino acid position. B. represent native residue glutamic acid, while structure C. represents mutant alanine residue designed by PDB ID: 1462171

These sequences were fetched to chimera by protein PDB IDs. (Figure 6)

Discussion

This study investigated the association between VCAM1 T-1594 C gene polymorphism and the risk of ischemic stroke in a group of Sudanese patients with sickle cell anaemia with history of stroke. It also looked at the role of VCAM1G -1238 C gene polymorphism in the protection from stroke in another group of patients with no history of stroke. Adhesion molecules, such as VCAM-1, regulate the attachment and migration of leukocytes and play a dominant role in the development of vascular disease in the general population[24]. Endothelial VCAM1 has been shown to be up-regulated in response to sickle erythrocytes and appears to be involved in the pathophysiology of microvascular occlusion in sickle cell disease.[25]-[27]. Dworkis et al strongly linked VCAM1 T 1594 C (rs1041163) with the SCA severity [20] and Hope et al suggested a strong association between VCAM1 (-1594) C and the risk of stroke [19]. However, in this study only 52.5% of stroke patients showed this mutation, this weak relation was supported by Fasano et al, findings who studied the same SNP seeking for possible association among stroke patients of SCD and came out with no association.[22] As recorded Some 1% of single base-pair substitutions causing human genetic disease occur within gene promoter regions, considering ours VCAM1 T 1594 C (rs1041163), where they disrupt the normal processes of gene activation and transcriptional initiation and usually decrease or increase the amount of mRNA and thus protein. Promoter mutations can alter or abolish the binding capacity of cis-acting DNA-sequence motifs for the trans-acting protein factors that normally interact with them.[28] Further studies are needed to specify these effects among the vcam1 gene different pathways. All our non-stroke patient's samples were run into RFLP-PCR except for 20 patients whose samples were sent for sequencing due to technical difficulties. The RFLP-PCR results showed the absence of the (VCAM-1) 1238C polymorphism. Sequencing results showed only two patients with the mutant (VCAM-1) 1238G > C polymorphism suggesting no association between the polymorphism and protection from stroke. This result goes with recently published results which revealed no association between the polymorphism and stroke.[21]Another more recent publication reported the presence of the wild type (VCAM-1) 1238G in high frequency among their population reveling no association with the protection from stroke.[22]Also using the bioinformatics tools showed variation among the predicted outcomes resulting from the presence of the mutant type. Using chimera software confirmed the reported outcomes of Boris et al. that the mutation of nonsynonymous 1238G >C SNP leads to a conservative amino acid change (Gly >Ala) at the 413th position in the immunoglobulin domain 5 of VCAM1 molecule. This

molecule has a large surface of interaction with the corresponding receptor, and thus, the weakly interacting methyl group added by substitution of glycine with alanine would not interfere with the proper function of the protein; therefore, the variant 413A may remain fully functional[21]. Further bioinformatics analyses using Polyphen2 and PHD-snpsoft wares predicted that the protein function would not be harmed the same findings that were reported by others who used MutPred and PolyPhen2 algorithms[29],[30]. Furthermore, the 413A VCAM-1 variant showed neither any potential difference in protein isoform levels nor any impairment of in vitro cell–cell adhesion function[31]. All these findings disagreed with results obtained from a study with a relatively small sample of SCA patients with clinically overt stroke who documented a protective association between the VCAM1 1238C variant and stroke[14] This is the fourth study worldwide to assess the effect of VCAM1 c.1238G>C polymorphism on the risk of stroke in children with SCA. Three of them confirmed our findings and only one said the opposite. Additional studies in different populations will be needed to confirm our findings. Although we examined only two SNPs of the VCAM1 variant in our group, other SNPs in linkage disequilibrium at the VCAM1 locus were found to contribute as a "haplotype" to the development of stroke[22] and need to be explored in our population.

Conclusions

The results of our study reveals the presence of VCAM1 (-1594) C variant among almost half of our stroke patients and not all of them which does not indicatea strong association between VCAM1 1594T > C genetic polymorphism and stroke. We also concluded that there is no protective association between G -1238 C genetic polymorphism and the non-stroke patients from the absence of G -1238 C SNP among the majority of the non-stroke patients. The complex aetiology of stroke suggests that individual genetic polymorphisms have modest effects that are difficult to detect, as has been observed to date, therefore larger studies are needed to assess these genetic polymorphisms as risk factors or protective modifiers. Also using the advanced bioinformatics tools as a predictive diagnostic tool may help enlightening the subsequent work in this field.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TA and BK and HA carried out the molecular genetic studies. TA, HA AF,MO and BK participated in the bioinformatics analysis. AK, BK BA and TA participated in the design of the study. TA, AK and BK participated in the coordination, and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by the University of Khartoum, Faculty of medical laboratory Sciences, Department of Haematology in part of PhD grant of Student Tyseer Alabid Elsiddig.

References

[1]. 1. Stuart, M.J. and B.N. Setty, Sickle cell acute chest syndrome: pathogenesis and rationale for treatment. Blood, 1999. 94(5): p. 1555-60.
[2]. 2. Ohene-Frempong, K., et al., Cerebrovascular accidents in sickle cell disease: rates and risk factors. Blood, 1998. 91(1): p. 288-94.
[3]. 3. Balkaran, B., et al., Stroke in a cohort of patients with homozygous sickle cell disease. J Pediatr, 1992. 120(3): p. 360-6.
[4]. 4. Rodgers, G.P., E.C. Walker, and M.J. Podgor, Is "relative" hypertension a risk factor for vaso-occlusive complications in sickle cell disease? Am J Med Sci, 1993. 305(3): p. 150-6.
[5]. 5. Styles, L.A., et al., Evidence for HLA-related susceptibility for stroke in children with sickle cell disease. Blood, 2000. 95(11): p. 3562-7.
[6]. 6. Tang, D.C., 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(3): p. 164-9.
[7]. 7. Ariyaratnam, R., et al., Genetics of ischaemic stroke among persons of non-European descent: a meta-analysis of eight genes involving approximately 32,500 individuals. PLoS Med, 2007. 4(4): p. e131.
[8]. 8. Ou, W., et al., Association of CVD candidate gene polymorphisms with ischemic stroke and cerebral hemorrhage in Chinese individuals. PLoS One, 2014. 9(8): p. e105516.
[9]. 9. Matarin, M., et al., Candidate gene polymorphisms for ischemic stroke. Stroke, 2009. 40(11): p. 3436-42.
[10]. 10. Ahmad, M., et al., Inflammation in ischemic stroke: mechanisms, consequences and possible drug targets. CNS Neurol Disord Drug Targets, 2014. 13(8): p. 1378-96.
[11]. 11. Suzuki, Y., et al., Involvement of Mincle and Syk in the changes to innate immunity after ischemic stroke. Sci Rep, 2013. 3: p. 3177.
[12]. 12. Swerlick, R.A., et al., Regulation of vascular cell adhesion molecule 1 on human dermal microvascular endothelial cells. J Immunol, 1992. 149(2): p. 698-705.
[13]. 13. Duits, A.J., et al., Enhanced levels of soluble VCAM-1 in sickle cell patients and their specific increment during vasoocclusive crisis. Clin Immunol Immunopathol, 1996. 81(1): p. 96-8.
[14]. 14. Taylor, J.G.t., et al., Variants in the VCAM1 gene and risk for symptomatic stroke in sickle cell disease. Blood, 2002. 100(13): p. 4303-9.
[15]. 15. Zameer, A. and S.A. Hoffman, Increased ICAM-1 and VCAM-1 expression in the brains of autoimmune mice. J Neuroimmunol, 2003. 142(1-2): p. 67-74.
[16]. 16. Kloda, K., [Associations of ICAM1 gene and VCAM1 promoter polymorphisms with transplanted kidney function]. Ann Acad Med Stetin, 2011. 57(1): p. 65-72.

[17]. 17. McDonnell, G.V., et al., Serum soluble adhesion molecules in multiple sclerosis: raised sVCAM-1, sICAM-1 and sE-selectin in primary progressive disease. J Neurol, 1999. 246(2): p. 87-92.
[18]. 18. Miyoshi, T., Z. Yuan, and W. Shi, Association of a Vcam1 mutation with atherosclerosis susceptibility in diet-induced models of atherosclerosis. Atherosclerosis, 2008. 196(1): p. 234-9.
[19]. 19. Hoppe, C., et al., Gene interactions and stroke risk in children with sickle cell anemia. Blood, 2004. 103(6): p. 2391-6.
[20]. 20. Dworkis, D.A., et al., Severe sickle cell anemia is associated with increased plasma levels of TNF-R1 and VCAM-1. Am J Hematol, 2011. 86(2): p. 220-3.
[21]. 21. Belisario, A.R., et al., Association of alpha-thalassemia, TNF-alpha (-308G>A) and VCAM-1 (c.1238G>C) gene polymorphisms with cerebrovascular disease in a newborn cohort of 411 children with sickle cell anemia. Blood Cells Mol Dis, 2015. 54(1): p. 44-50.
[22]. 22. Fasano, R.M., E.R. Meier, and M.L. Hulbert, Cerebral vasculopathy in children with sickle cell anemia. Blood Cells Mol Dis, 2015. 54(1): p. 17-25.
[23]. 23. Altschul, S.F., et al., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res, 1997. 25(17): p. 3389-402.
[24]. 24. Cybulsky, M.I., et al., A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest, 2001. 107(10): p. 1255-62.
[25]. 25. Shiu, Y.T., M.M. Udden, and L.V. McIntire, Perfusion with sickle erythrocytes up-regulates ICAM-1 and VCAM-1 gene expression in cultured human endothelial cells. Blood, 2000. 95(10): p. 3232-41.
[26]. 26. Gee, B.E. and O.S. Platt, Sickle reticulocytes adhere to VCAM-1. Blood, 1995. 85(1): p. 268-74.
[27]. 27. Belcher, J.D., et al., Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion. Blood, 2000. 96(7): p. 2451-9.
[28]. 28. de Vooght, K.M., R. van Wijk, and W.W. van Solinge, Management of gene promoter mutations in molecular diagnostics. Clin Chem, 2009. 55(4): p. 698-708.
[29]. 29. Li, B., et al., Automated inference of molecular mechanisms of disease from amino acid substitutions. Bioinformatics, 2009. 25(21): p. 2744-50.
[30]. 30. Adzhubei, I.A., et al., A method and server for predicting damaging missense mutations. Nat Methods, 2010. 7(4): p. 248-9.
[31]. 31. Schmitz, B., et al., Increased monocyte adhesion by endothelial expression of VCAM-1 missense variation in vitro. Atherosclerosis, 2013. 230(2): p. 185-90.

Appendix: Accession numbers for some of the sequenced samples of the study

BankItvac 1896808 Sequence ID	Accesion number
VAC-1	**KU759842**
VAC-2	**KU759843**
VAC-3	**KU759844**
VAC-4	**KU759845**
VAC-6	**KU759846**
VAC-7	**KU759847**
VAC-8	**KU759848**
VAC-9	**KU759849**
VAC-10	**KU759850**
VAC-11	**KU759851**
VAC-12	**KU759852**
VAC-13	**KU759853**
VAC-14	**KU759854**
VAC-15	**KU759855**
VAC-16	**KU759856**
VAC-17	**KU759857**
VAC-18	**KU759858**
VAC-19	**KU759859**
VAC-20	**KU759860**
VAC-21	**KU759861**
BankIt1893312 Pro Sequence ID	**Accession number**
Pro-1	**KU705816**
Pro-2	**KU705817**
Pro-3	**KU705818**
Pro-4	**KU705819**