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Cystathionine Beta Synthase *(CBS)* Gene 844ins68 Polymorphism in Sickle Cell Disease Patients: Frequency of Vaso-Occlusive Crisis

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ABSTRACT

Background: Sickle cell disease is (SCD) is a chronic hereditary hemolytic anemia characterized by a hypercoagulable and inflammatory state which can lead to vaso-occlusive episodes. Increased serum homocysteine level is an independent risk factor for thrombo-embolism and cardiovascular disease and is therefore of interest in sickle cell disease. The 844ins68 mutation/polymorphism, occurring in the cystathionine beta synthase (CBS) gene which controls the CBS enzyme activity, is accompanied by hyper-homocysteinemia. This mutation, in homozygous or heterozygous state, lowers CBS enzyme activity and is thus considered an independent risk factor for artery occlusion.

Objectives: To determine the frequency of the 844ins68 CBS gene mutation/polymorphism and its contribution to hyper-homocysteinemia and vaso-occlusive episodes in sickle cell disease and sickle/beta ß thalassemia (Sß-thalassemia) patients.

Patients and Methods: Nineteen sickle cell disease, 32 Sß-thalassemia and 2 sickle trait subjects together with 42 age and sex matched healthy controls (HCs) were included. Fasting serum homocysteine level was measured using immunenephelometry. The *CBS* 844ins68 mutation/polymorphism was detected using conventional polymerase chain reaction (PCR).

Results: The frequency of the CBS gene wild type was 78.8%, 73% and 81.3% in the HCs, SCD and Sß-thalassemia groups respectively. The heterozygous variant was observed in 19%, 28.3% and 15.6% respectively. The homozygous gene was detected only in the HCs and Sß-thalassemia groups at a rate of 2% and 3.1% respectively. A significant increase in fasting serum homocysteine level was found in subjects with either homozygous or heterozygous variants compared to wild type subjects (p=<0.001). A significant increase in the frequency of vaso-occlusive crisis (VOC) was found in SCD patients exhibiting this variant (p=0.05). Positive correlation was found between fasting serum homocysteine level and frequency of VOC (r=0.30, p=0.03). Positive correlation was also found between age and fasting homocysteine level (r=0.36, p=0.009).

Conclusion: The 844ins68 mutation/polymorphism of the CBS gene is a risk factor for VOC and hyper-homocysteinemia in sickle cell disease.

Key Words: *Sickle Cell Disease – Vaso-occlusive crisis – Cystathionine beta-synthase gene – Hyper-homocysteinemia.*

INTRODUCTION

Sickle cell Disease (SCD) is a hemoglobin disorder with an evident inflammatory picture resulting in activated endothelial cells, with increased adhesion of platelets, leucocytes and sickled red cells to the endothelium leading to vaso-occlusive episodes **[1]**. SCD patients present with a diverse disease phenotypes where some show higher risk for morbidity and mortality than others. This suggests the importance to study factors that may contribute to aggravating these events. Thrombotic occlusion of both superficial and deep veins is considered the most important event in SCD pathophysiology and may explain some of the disease clinical manifestations **[2]**. Cystathionine beta-synthase (CBS) acts as a critical enzyme in homocysteine metabolism converting homocysteine to cystathionine, with pyridoxal phosphate as a cofactor. Previous literature has reported an association between CBS enzyme deficiency, hyper-homocysteinemia and thrombotic events **[3]**. The coding gene is found on

chromosome 21 and consists of 23 exons **[4]**. The most common CBS gene variation, resulting in enzyme deficiency is the 844ins68 polymorhism **[5]** occurring on exon 8 **[6]**.

In this work, we studied the frequency of the CBS gene 844ins68 mutation / polymorphism in SCD and sickle / beta thalassemia (Sß-thalassemia) and evaluated its possible contribution to hyper-homocysteinemia and vaso-occlusive episodes.

PATIENTS AND METHODS

A case control study was conducted on 53 patients (19 SCD, 32 Sß-thalassemia and 2 sickle traits) recruited from Cairo University and Fayoum University Hospitals. Patients included 23 males (43.3%) and 30 females (56.6%) with an age range of 3-36 years, median of 12 years and a mean 14.1±8.2 years. Forty two age and gender matched healthy individuals were included as controls. All subjects/guardians signed an informed consent and the Fayoum Faculty of Medicine research ethics committee approved the study. Exclusion criteria included those less than 2 years of age, diabetics, severe concurrent disease, kidney disease, smokers, and those who had received blood transfusion within a month of sampling. Full history was taken with emphasis on smoking and dietary habits and disease history (disease onset, severity and frequency of vaso-occlusive crisis (VOC) and blood transfusion). Based on criteria reported by Darbari et al., **[7]** and Acipayam et al., **[8]** to describe the intensity of the acute pain of VOC, patients were sub-grouped into those suffering from mild pain (Group I) and those suffering from moderate to severe pain (Group II) (Table 1). Following full history taking and complete examination, overnight fasting samples were collected. Fasting serum homocysteine level was measured by immunonephelometry on the Immulite 1000 using Immulite homocysteine kit, catalog #LKH01 (Siemens Healthcare Diagnostics). Normal fasting serum homocysteine level is up to 12µmole/L **[9]**. Genomic DNA was purified using spin columns (Gene JET Blood Ge-nomic DNA purification Kit, Catalog # K0721, Fermentas, USA). The CBS gene 844 ins 68 polymorphism was detected by conventional PCR using 5' CTGGCCTTGAGCCCTGAA 3' and 5' GGCCGGGCTCTGGACTC 3' sense and anti-sense primers respectively **[10]** (Fermentas, USA). The PCR reaction mix consisted of 12.5 µl ready to use Taq PCR mas-ter mix kit (Qiagen, Germany), 1µl of each primer (25 pmole working concentration), 1.5-3µl of DNA template (150-300ng) and nuclease free water in a total volume of 25µl. Amplification was done using a Hybaid PCR express 01747-UK thermal cycler programmed as follows: Initial denaturation at 95ºC for 3 minutes; 30 amplification cycles consisting of denaturation at 95ºC for 1min, annealing at 60ºC for 1min, extension at 72ºC for 2min; and finally an additional extension cycle at 72ºC for 3min. Detection of the PCR product was done on an ethidium bromide stained 2% agarose gel. A stained ready for use 100 base pair DNA molecular weight marker from Fermentas, USA was run simultaneously with the test PCR products. The wild type gave a single band at 184 base pair (bp). The heterozygous samples gave 2 bands: The 184bp band and an additional 252 band containing the 64 bp insertion. The homozygous samples gave a single 252 bp band (Fig. 1).

RESULTS

Among controls, 33 (78.6%) had wild type of the CBS gene 844 ins 68 mutation/polymorphism, 8 (19%) were heterozygous and only one was homozygous (2%). Among SCD patients, 14 (73%) had wild type, 5 (26.3%) were heterozygous and none were homozygous. Among sickle ß-thalassemia patients, 26 (81.3%) were wild type, 5 (15.6%) heterozygous and only one (3.1%) was homozygous for the 844 ins 68 mutation. Comparison of the different *CBS* genotypes among controls and patients showed no statistically significant difference (p=0.08). There was positive consanguinity in 34.7% of our study group. The frequency of the CBS 844 ins 68 mutation was corrected for consanguinity **[11]**.

Fasting homocysteine level reached significantly higher value (20µmole) in the patient homozygous for 844 ins 68 mutation when compared with both the heterozygous patients and the wild type patients (9.9±2.4 and 7.8± 2.9µmol/l respectively, p=<0.001). This homozygous patient showed the highest frequency of VOC (12 times/year) when compared to heterozygous and wild (p=0.05). A significant positive correlation was found between the

fasting Hcy level and the frequency of VOC (r=0.30 & p=0.03, Fig. 2), and age (r=0.36 and p=0.009) but no correlation was found with determinants of severity of VOC (Table 2).

There was neither association between VOC and patient groups (SCD and sickle/beta thalassemia (p=0.2), nor correlation with Hb S level (r=–0.13; p=0.3). Neither was there an association between *CBS* genotypes and VOC severity (p=0.6).

Both, the frequency of the CBS gene mutation and the serum homocysteine levels, were comparable between cases with and without other disease complications (acute chest syndrome, avascular necrosis, deep vein thrombosis and CNS complications) (Table 3). The association of CBS genotypes with the severity of the VOC is presented in Table (4).

Table (1): Classification of 53 sickle cell disease patients by vaso-occlusive crisis severity.

Measuring Criteria of intensity of pain	Severity of pain	
Verbal description	Slight, mild	Moderate, severe, very severe pain
Pain relief	Spontaneous relief or home administration of analgesics	Need of emergency department visits or hospitalization
Effect on daily activity	No or mild effect	Marked affection of activity (absence from school or work)

Table (2): Correlation between fasting homocysteine levels and age, vaso-occlusive crisis frequency and determinants of severity in 53 sickle cell disease patients.

Variable	Homocysteine level	
	r	*p*
Age: Years	0.36	0.009
Age of onset of SCD: Years.	0.032	0.8
Age of onset of blood transfusion: Years	–0.06	0.7
Frequency of blood transfusion /year.	0.24	0.08
Frequency of VOC crisis/year.	0.30	0.03

Table (3): Comparison of CBS genotypes and homocysteine levels between sickle cell disease patients with and without complications.

Parameter	Complications n=7	No complications n=46	*p*-value
CBS genotype: No (%):			
Wild type	6 (85.7%)	36 (78.3%)	
Heterozygous	1 (14.3%)	9 (19.6%)	0.8
Homozygous	0 (0%)	1 (2.2%)	
Homocysteine level: µmol/l:			
Mean ± SD	9.2±3.8	8.4±3.2	0.5

Table (4): Comparison of crisis severity among sickle cell disease patients with different CBS genotypes.

Crisis severity	Wild type (No=42)		Heterozygous (No=10)		Homozygous (No=1)	
	No.	%	No.	%	No.	%
No crisis: No=4	3	7.1	1	10	0	0
Mild crisis: No=32	27	64.3	4	40	1	100
Severe crisis: No=17	12	28.6	5	50	0	0
p-value	0.6					

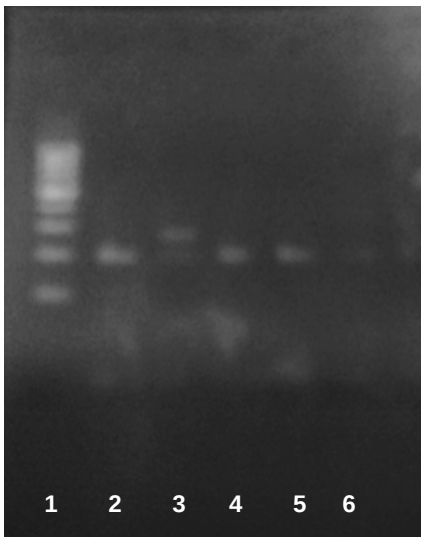

Fig. (1): Ethidium bromide-stained agarose gel of CBS 844ins68 mutation.

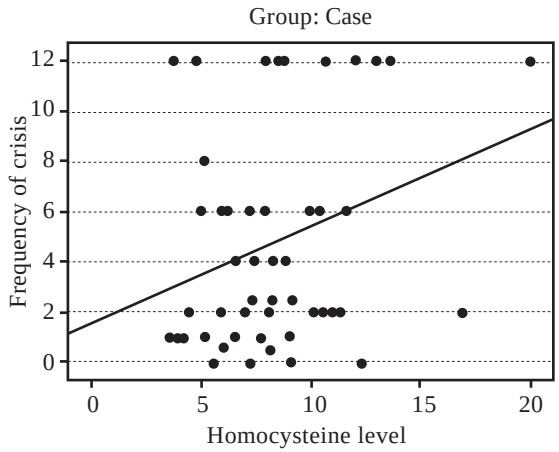

Fig. (2): Correlation between frequency of vaso-occlusive crisis and homocysteine level in 53 sickle cell disease patients (r=0.3, p=0.03).

DISCUSSION

Sickle cell disease (SCD) is a worldwide hemoglobin disorder characterized by chronic hemolysis and recurrent ischemia due to micro-vascular occlusion following the adhesion of erythrocytes and leukocytes to the vascular endothelium. In addition, SCD is compli-cated by chronic coagulation and endothelial activation resulting in a hyper-coagulable state **[12]**.

Periodic episodes of severe pain known as vaso-occlusive crises (VOC) are the most common symptom of SCD, adversely impacting the quantity of life of the patients and care givers. However, there is a large inter-patient variability in the frequency and severity of vaso-occlusive crises **[8]**.

Homocysteine is a sulfhydryl amino acid derived from the metabolic conversion of methionine, which is dependent on vitamins (folic acid, B12 and B6) as cofactors or sub-strates. Hyper-homocysteinemia is a well-known culprit in many patients suffering a hyper-coagulable state. Many case-control, cross-sectional and several epidemiological studies have demonstrated that mild to moderate hyper-homocysteinemia is an important risk factor for cerebral artery disease, occlusive arterial disease, coronary artery disease and thrombo-embolism **[13,14]**.

Hyper-homocysteinemia is proved to be multi-factorial including nutritional, genetic and lifestyle factors. Studies examining the different possibilities have discovered a number of gene variants of functional enzymes in the homocysteine metabolism **[14]**. Cystathionine beta synthase *(CBS)* is a critical enzyme in the trans-sulfuration pathway in homocysteine metabolism, converting homocysteine to cystathionine with pyridoxal phosphate (B6) as an important cofactor. Abnormalities of the CBS gene may result in enzyme deficiency and hyper-homocysteinemia **[16]**.

This case-control study aimed to evaluate the frequency of 844ins68 of the CBS gene and its correlation with hyper-homocysteinemia and vaso-occlusive crisis in sickle cell disease patients.

The study included 95 subjects ; 53 sickle cell disease patients and 42 age and gender matched apparently healthy individuals with normal blood picture and hemoglobin electrophoresis.

The presence of mutant allele of CBS gene showed slight insignificant higher frequency in SCD patients (14.1%) than in controls (12.8%). This is slightly less than the results obtained by Kluijtmans et al who reported a 17.7% frequency for his Irish study population which was comparable with the literature for this genotype **[15]**.

There was no statistically significant difference in fasting homocysteine (Hcy) levels be-

tween SCD patients and controls (*p*-value=0.8) which is in agreement with Segal et al., **[17]**.

In this study, heterozygous or homozygous CBS 844ins68 mutation was associated with significant increase in fasting serum Hcy (*p*-value=0.001). This is in agreement with Elsaid et al., **[18]** who reported significantly increased levels of fasting Hcy in heterozygous parents of patients with homocysteinuria (*p*=0.012). Studying the genetic determinants of intermediate and severe hyper-homocysteinemia (HHcy) with thrombosis, Gaustadnes et al., **[19]**, reported severe HHcy in patients with co-existing MTHFR T/T genotype and CBS compared to moderate HHcy in the latter alone.

In this study, VOC was more frequent in patients heterozygous and homozygous for CBS 844ins68 genotype compared to those with wild type allele (*p*-value=0.05); the highest frequency was demonstrated in the single homozygous patient (12 times per year). This is in agreement with the results of Jacob et al., **[20]**, who reported that the presence of the *CBS* 844ins68 genotype is a risk factor for vaso-occlusive episodes in SCD. However, severity of VOC was not significantly different among the different genotypes in our study (*p*-value =0.6).

The current study showed a significant positive correlation between fasting Hcy level and the frequency of VOC (*r*=0.03, *p*-value=0.03) and age (*r*=0.36, *p*-value=0.009). To date the mechanisms of vascular injury due to HHcy are not fully understood. HHcy enhances oxidative stress-induced vessel wall inflammatory cell differentiation and maturation leading to atherosclerosis **[21]**. HHcy also causes vascular injury through intracellular homocysteine-induced protein modification in the endoplasmic reticulum, known as endoplasmic reticulum stress. This results in dysregulation of lipid metabolism, activation of inflammatory pathways and apoptotic cell death **[22]**. Moreover, it up-regulates pathogenic genes and down regulates protective genes via interference with transferring methyl group metabolism (demethylation and methylation). It also contributes to vascular injury by modulating the intracellular redox state and altering protein function **[20]**. HHcy causes inhibition of thrombomodulin-induced activation of protein C **[22]**, and impairs the ability of activated protein C to activate its major substrate, FVa **[24]**.

The increasing Hcy levels with age may be explained by increasing deterioration of its renal elimination by the tubular cells. Also it is due to age related decrease in the activity of enzymes involved in its elimination from plasma **[25]**. Significant positive correlation between age and Hcy levels was reported by a number of studies **[25,26]**.

In conclusion, the current study showed that the distribution of CBS 844ins68 genotypes is comparable between SCD patients and normal controls. However, the presence of the 844 ins 68 mutation in the CBS gene was accompanied by higher fasting levels of homocysteine and increased risk for vaso-occlusive episodes. Significant positive correlation was found between homocysteine level and both age and the frequency of VOC crisis.

REFERENCES

1- De Franceschi L. Pathophysiology of sickle cell disease and new drugs for the treat-ment. Mediterr J Hematol Infect Dis. 2009; 20 e2009024.

2- Ware RE, de Montalembert M, Tshilolo L, Abboud MR: Sickle cell disease. Lancet. 2017; 390: 311-322.

3- Blom HJ. Genetic determinants of hyperhomocystinemia: The roles of cystathionine beta-synthase and 5,10- methyltetrahydrofolate reductase. Eur J Pediatr. 2000; 159 S: 208-212.

4- Kraus JP, Janosik M, Kozich V et al., Cystathionine beta-synthase mutations in homo-cysteinuria. Hum Mutat. 1999; 13: 362-375.

5- Hobbs CA, Sherman SL, Yi P. Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome. Am J Hum Genet. 2000; 67: 623-630.

6- Tsai MY, Bignell M, Schwitchenburg K, Hanson NQ. High prevalence of a mutation in the Cystathionine b-Synthase gene. Am. J. Hum. Genet. 1999; 59: 1262-1267.

7- Darabi DS, Onyekwere O, Nouraie M et al. Markers of severe vaso-occlusive painful episode frequency in children and adolescents with sickle cell anemia. J Pediatr. 2012; 160: 286-290.

8- Acipayam C, Dorum BR, Ilhan G, Ersoy A, Oktay G, Helvaci MR. Predictive value of pain intensity in the clinical severity of painful crises in children and adolescents with sickle cell disease. ME-JIM. 2014; 7: 28-33.

9- Plazar N, Jurdana M. Hyperhomocysteinemia: Relation to cardiovascular disease and venous thromboembolism, pathophysiology and clinical aspects of venous

throm-boembolism in neonates, renal disease and cancer patients,Dr. Mohamed A. Abdelaal (Ed.) 2012; 17-34, ISBN: 978-953-51-0616-6, InTech, Available from: http://www.intechopen.com/books/ pathophysiology-and-clinical-aspects-of-venous- thromboembolism-in-neonates-renal-disease-and-cancer-patients/hyperhomocysteinemia-relation-to- cardiovascular-disease-and-venous-thromboembolism.

10- Tsai MY, Bignel M, Schwichtenberg K, Hanson NQ. High prevalence of a mutation in the cystathionine beta-synthase gene. Am J Hum Genet 1996; 59: 1262-1267.

11- Yonker MA, Teeuw ME, Ten Kate LP. An improved method for estimating total patho-genic allele frequency of autosomal recessive disorders when parents are consan-guineous, http://research.vu.nl/ws/portalfiles/portal/1298035.

12- Beers E, Schaap M, Berckman R, et al. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. Hematologica. 2009; 94: 1513-1519.

13- Cattaneo M. Hyperhomocysteinemia, atherosclerosis and thrombosis. Thromb Hae-most. 1999; 81: 165-176.

14- Cattaneo M. Hyperhomocysteinemia and thrombosis. Lipids. 2001; 36 (Suppl: S13-26).

15- Kluijtmans L, Young L, Boreham C, et al. Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults. Blood. 2003; 101: 2483-2488.

16- Janosik M, Kery V, Gaustadnes M, Mclean KN, Kruas JP. Regulation of human cys-tathionine beta-synthase by S-adenosyl-L-methionine evidence for two catalytically ac-tive conformations involving an auto inhibitory domain in the C-terminal region. Biochemistry. 2001; 40: 10625-10633.

17- Segal J, Miller E, Brereton N, Reer LM. Concentrations of B vitamins in children with sickle cell anemia. South Med J. 2004; 97: 149-155.

18- Elsaid M, Bener A, Lindner M, et al. Are heterozygotes for classical homocysteinuria at risk of vitamin B12 and folic acid deficiency? Mol Genet Metab. 2007; 92: 100-103.

19- Gaustadnes M, Rüdiger N, Rasmussen K, Ingerstev J. Intermediate and severe hyper-homocysteinemia with thrombosis: A story of genetic determinants. Thromb Haemost. 2000; 83: 554-548.

20- Jacob M, Bastos C, Bonini-Domingos C. The 844ins68 cystathionine beta-synthase and C677T MTHFR gene polymorphism and the vaso-occlusive event risk in sickle cell disease. Arch Med Sci. 2011; 7: 97-101.

21- Fu Y, Wang X, Kong W. Hyperhomocysteinemia and vascular injury: The advance of mechanisms and drug targets. Br J Pharmacolol. 2017; Aug 24. doi:10.111 /bph. 13988.

22- Lentz SR. Mechanisms of homocysteine-induced atherothrombosis. Thromb Hemost. 2005;3:1646-1654.

23- Dayal S, Bottiglieri T, Arning E, et al. Endothelial dysfunction and elevation of S-adenosylhomocysteine in cystathionine B-synthase-deficient mice. Circ Res. 2001; 88: 1203-1209.

24- Undas A, Williams EB, Butenas S, Orfeo T, Mann KG. Homocysteine inhibits activation of Factor Va by activated protein C. J Biol Chem. 2001; 276: 4389-4397.

25- Straßburg A, Krems C, Lührmann M. Effect of age on plasma homocysteine concentra-tions in young and elderly subjects considering serum vitamin concentrations and dif-ferent lifestyle factors. Int J Vitam Nutr Res. 2004; 74: 129-136.

26- El-Sammak M, Kandil M, El-Hifni S, Hosni R, Ragab M. Elevated plasma homocysteine is positively associated with age independent of C677T mutation of the methylenetet-rahydrofolate reductase gene in selected Egyptian subjects. Int J Med Sci. 2004; 1: 181-192.