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ORIGINAL ARTICLE

Association of plasma homocysteine level with vaso-occlusive crisis in sickle cell anemia patients of Odisha, India

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Abstract

Vascular complications of sickle cell anemia (SCA) are influenced by many factors. Elevated plasma homocysteine (Hcy) is supposed to be an independent risk factor and is either genetic or nutritional origin. The present study evaluated the plasma Hcy level, MTHFR C677T gene polymorphism, effect of folic acid (FA) supplementation, and hemato-biochemical parameters in SCA and their effect on the vaso-occlusive crisis (VOC) in SCA patients of an Asian-Indian haplotype population. One hundred twenty cases of SCA (HbSS) and 50 controls with normal hemoglobin(HbAA) were studied. It was found that the plasma Hcy level is significantly higher ($p < 0.0001$) in patients with SCA (22.41 ± 7.8 μmol/L) compared to controls (13.2 ± 4.4 μmol/L). Moreover, patients without FA supplementation had a significantly ($p < 0.001$) higher Hcy level (27 ± 7 μmol/L) compared to those with supplementation (17.75 ± 5.7 μmol/L). Turkey-Kramer multiple comparison tests show that there is a significant difference ($p < 0.05$) in HbF percent, hemoglobin (Hb), platelet count, serum bilirubin (direct:Bil-D and total:Bil-T), aspartate transaminase (AST), lactate dehydrogenase (LDH), and plasma Hcy levels between mild and severe VOC. Between moderate VOC and severe VOC, there was a significant difference ($p < 0.05$) in HbF%, Bil-D, AST, Hcy. Pearson correlation revealed that plasma Hcy had a significantly ($p < 0.05$) positive correlation with AST, serum bilirubin (indirect and total), LDH, jaundice, stroke, VOC per year, and hospitalization per year whereas it was inversely correlated with HbF percentage, Hb level, and FA treatment. In the study population, increased plasma Hcy level, hemolysis, and platelet activation were found to influence VOC in SCA.

Keywords Sickle cell anemia · Homocysteine · Folic acid · Vaso-occlusive crisis · Hemolysis · Methyltetrahydrofolate reductase

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Introduction

Sickle cell anemia (SCA) is an autosomal recessive disease caused due to transversion mutation (A > T) on the 6th codon of the β-globin gene leading to the change of amino acid from glutamic acid to valine [1]. In hypoxic condition, valine exposed to the surface forms a hydrophobic bond with the nearest valine of other β-globin chain and forms a polymer impelled internally to change a normal RBC into a sickle-shaped RBC (SRBC) [2]. Sickle RBC becomes sticky, rigid, irreversible, irregular shaped, and with activated platelet, leukocyte, and endothelial membrane can get stuck into small blood vessels, which can slow down or block blood flow to different parts of the body leading to vaso-occlusion at that site [3–5]. In SCA, clinical presentation varies from asymptomatic to severe symptoms affecting many organs and even premature death. The vaso-occlusive crisis (VOC) episodes are the most common complication, requiring medical attention [6].

Intravascular hemolysis of RBC plays a key role in the development of VOC in SCA patients [7]. Briefly, on hemolytic release of free hemoglobin into plasma inactivates the nitric oxide (vasodilator) and oxidizes hemoglobin to form methemoglobin which releases the heme moiety. The free heme induces production of reactive oxygen species (ROS) and inflammation, leading to the activation of platelets and neutrophils and forms a sticky environment in the endothelium.

Owing to the short life span of SRBC and hemolytic environment, the erythropoietic mechanism demands more folate, which possibly relates to elevated homocysteine (Hcy) level [8–11]. Homocysteine is formed at an intermediate step in methionine and cysteine biosynthesis and has a key role in vasculopathy [12, 13]. Homocysteine triggers the collagen type I protein, which induces platelet activation through signaling components of glycoprotein VI and integrin α2β1 pathway [14]. Once activated, the platelets become stickier due to the expression of the P-selectin and glycoprotein IIb/IIIa and adhere to the endothelial membrane [4, 15]. This might allow relating a reportedly high level of Hcy in SCA patients [11, 16, 17]. Hyperhomocysteinemia (hHcy) is an independent risk factor of the vascular disease, leading to ischemic complications and atherosclerosis [16, 18, 19] and is associated with the C677T gene polymorphism (rs1801133) in the methylene tetrahydrofolate reductase (MTHFR) gene [20]. The C677T gene polymorphism is also involved in Hcy metabolism. It has been observed that the MTHFR enzyme activity reduces by almost 35% in the heterozygous state (CT) and by approximately 70% in the homozygote state (TT) of the C677T gene polymorphism [21].

High fetal hemoglobin (HbF) has a protective role by causing hindrance to the polymerization process which prevents the formation of the sickle shape, and due to high affinity towards oxygen, it makes the SRBC more oxygenated and help to maintain normal shape. Also, alpha thalassemia has been reported to reduce the concentration of HbS and hence the polymerization [22–27].

Sickle cell disease is widespread in central India and in the western parts of Odisha, with a high sickle allele frequency [6, 28–30]. Indian SCA patients postulated to have an Arab-Indian (AI) haplotype background have reportedly higher HbF concentration [22]. In spite of high HbF, studies have reported that patients with SCA from Odisha suffer from the severe manifestation of VOC [31]. This has urged to find out the possibilities of involvement of other factors responsible for VOC in the studied population. There is a paucity of literature and lack of studies of Hcy in SCA in AI haplotype population. Pandey et al. show that Indian patients do have high Hcy but fail to show any clinical correlation with the observation of the high Hcy [11]. Hence, the present study was undertaken to find out the possible association between the levels of Hcy, genetic polymorphism of the C677T polymorphism in the MTHFR gene, and various biochemical and hematological parameters reflecting clinical severity in SCA patients from Odisha, India.

Materials and methods

Recruitment of study cases

This study was carried out between September 2015 and December 2018 at the Sickle Cell Institute, and Department of Medicine of Veer Surendra Sai Institute of Medical Science and Research (VIMSAR), Burla, and Fakir Mohan University, Balasore, Odisha, India. A total of 600 patient recruitment was done from the in-patient wards of medicine unit and sickle cell institute, VIMSAR. Written consent was availed from all the patients recruited before collection of blood samples and clinical data of the last 1 year. Sickling test, alkaline agarose gel electrophoresis (pH-8.6), and CE-HPLC analysis for quantifying different hemoglobin (Hb) components using variant II β-thalassemia short-program Hb testing system (Bio-Rad Laboratories, Hercules, CA, USA) were performed on all cases. Cases diagnosed with HbSβ −thalassemia, HbSD^{Punjab}, HbSE, and other compound heterozygotes; SCA patients below 15 years of age, smokers, and/or those suffering from diabetes mellitus, pulmonary hypertension, renal failure, hepatic failure, atherosclerosis, or continuing treatment with hydroxyurea, vasodilators; and those with pregnancy and malignancies were excluded from the study owing to the possible influence of these on the Hcy levels. Furthermore, the patients attending to the sickle cell outpatient department (OPD) with mild or moderate painful events were excluded who attended the unit after either breakfast or lunch.

Out of 600 cases, 170 cases were selected for this study. Among them, 120 cases belonged to homozygote SCA

(HbSS) and 50 cases were considered as normal healthy controls (HbAA). This study was approved by the institutional ethical committee of VIMSAR, Burla, Odisha (VIREC-No.2016/I-F-CT-01/008).

VOC categorization

Vaso-occlusive crisis may be defined as a painful event that may last for more than 4 h and requires analgesics for relief when no other cause can explain the symptoms [32]. Vaso-occlusive crisis in patients was categorized into three main groups in this study based on their pain severity, a modified version using visual analog scale, and pharmacological management of pain according to the World Health Organization three-step ladder. Mild VOC category was defined as a crisis in which patients feel mild pain and may need non-opioid with adjuvant. Moderate VOC was considered as a crisis which can be managed in outdoor care center with weak opioid (or low dose of strong opioid), non-opioid, and adjuvant. Severe VOC was considered when the patient needed to be hospitalized, and intensive care was required to relieve pain with a strong opioid, non-opioid, and adjuvant [33–35].

Laboratory investigation

Five milliliters of fasting blood samples was collected for the analyses from the patients who attended the OPD during the clinical check up and from indoor patients while in painful crisis. Separate blood samples were collected for CBC and molecular analysis (EDTA blood), for biochemical analysis (serum clot activator), and for Hcy estimation (in sodium fluoride medium). All samples were kept at 4 °C until analysis within 1 hour of collection. Plasma was separated by centrifugation (5000 rpm/5 min) and stored at 4 °C for the sample collected for Hcy estimation. Homocysteine was estimated within 24 hour of collection, and DNA was isolated within 1 month. The complete blood count (CBC) was performed using Sysmex KX 21 (Sysmex Corporation, Kobe, Japan). Serum bilirubin (BIL-T and BIL-D), serum creatinine (CRT), aspartate transaminase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) were estimated on Cobas Integra 400 Plus (Roche Diagnostics Ltd., Rotkreuz, Switzerland), as per the manufacturer's instructions and standard protocol. The concentration of serum Hcy was determined by photometric analysis. Homocysteinemia classification was adopted as per Kang et al. (1992) and Refsum et al. (2004). Plasma total Hcy (ptH) was considered normal when it was below 15 μmol/L, moderate hyperhomocysteinemia (mhHcy) was considered when ptH value was between 15 and 30 μmol/L, and intermediate hyperhomocysteinemia (ihHcy) was considered when ptH value was between 30 and 100 μmol/L, and patients with ptH level above 100 μmol/L were recorded to have severe hyperhomocysteinemia (shHCY) [36, 37].

MTHFR C677T genotype investigation

Genomic DNA was extracted from the whole blood sample by the standard phenol-chloroform method [38]. The MTHFR C677T gene polymorphism was detected by PCR (198 bp) using a pair of primers (Forward: 5′-TGAA GGAGAAGGTGTCTGCGGGA-3′ and Reverse: 5′-AGGA CGGTGCGGTGAGAGTG-3′), followed by digestion with *Hinf* I. The digested product was separated on a 2% agarose gel and documented for specific sizes. Product size of only one 198 bp band was indicative of wild-type homozygote (CC), while presence of three bands: 198 bp, 175 bp, and 23 bp was recorded as the heterozygote (CT), and presence of two bands 175 bp and 23 bp was considered mutant homozygote (TT) (supplementary Fig. 1) [21].

Statistical analysis

For comparing normal controls (HbAA) and patients with SCA (HbSS), Student's *t* –test was performed using GraphPad InStat Version 3.0. Pearson correlation analysis and stepwise multiple regression analysis were performed using SPSS version 23.0 (Statistical Package for Social Sciences, Inc., Chicago, III) with the Hcy level among clinical, hematological, and biochemical variables after validation of normality. Level of significance was set at $p < 0.05$. Allele and genotype frequency of the cases and controls was calculated using SNPStarts online software [39], and chi-square (χ^2) test was performed to study the association of clinical presentation with the MTHFR genotype.

Results

Out of the 120 cases of SCA, 61 were males and 59 were females. The SCA patients had a median age of 23 for males and 24 for females. Healthy controls with normal genotype included 29 males and 21 females with a median age of 22 and 20 years respectively. Overall age ranged between 15 and 50 years. The mean ages among the male and females of both SCA cases and healthy controls were distributed equally ($p > 0.05$). However, the SCA cases had significantly higher HbF%, Bil-D, AST, ALT, LDH, and Hcy than that of the controls ($p < 0.05$) (Table 1). The history of complications and problems as informed by the patient for the past 1 year was recorded for all SCA cases, and it revealed that VOC was the commonest complain in this group (85%) and anemia being the second most common complain (74.17%) followed by jaundice (47.5%), fever (36%), and acute chest syndrome (ACS) (1%). We calculated that prior to registration with our

Table 1 Socio-hematological, biochemical, and clinical comparison between SCA and normal cases

Number of subjects	Mean ± SD		*p* –value
	Sickle cell anemia 120	*Control (Normal)* 50	
Mean age and range	24.03 ± 8.06 (15–50 yr)	21.15 ± 4.73 (15–50 yr)	> 0.05
Gender			
Male	61	32	> 0.05
Female	59	18	
HbA (%)	2.23 ± 0.72	86.64 ± 0.8	*< 0.001*
HbA2 (%)	2.49 ± 0.88	2.64 ± 0.2	> 0.05
HbS (%)	74.33 ± 5.64	–	–
HbF (%)	20.28 ± 5.94	0.52 ± 0.13	*< 0.001*
WBC ($\times 10^3/\mu L$)	10.1 ± 3.9	8.2 ± 2.2	> 0.05
RBC($\times 10^6/\mu L$)	3.05 ± 0.79	5.16 ± 0.76	*< 0.01*
Hb (g/dL)	8.3 ± 1.6	12.0 ± 1.9	*< 0.05*
HCT (%)	26.27 ± 6.9	40.76 ± 4.8	*< 0.001*
MCV (fL)	84.38 ± 11.3	88.38 ± 8.4	> 0.05
MCH (pg)	27.14 ± 3.5	29.37 ± 3.2	> 0.05
MCHC (g/dL)	32.38 ± 3.9	33.16 ± 1.21	> 0.05
Platelets ($\times 10^5/\mu L$)	2.67 ± 1.66	2.92 ± 0.86	> 0.05
Bilirubin-direct (mg/dL)	0.24 ± 0.15	0.12 ± 0.08	> 0.05
Bilirubin-total (mg/dL)	2.49 ± 1.6	0.95 ± 0.2	*< 0.001*
AST (U/L)	58.04 ± 21.1	16.9 ± 2.5	*< 0.001*
ALT (U/L)	56.8 ± 21.9	13.1 ± 3.7	*< 0.001*
LDH (U/L)	734.64 ± 540	184.8 ± 279.5	*< 0.001*
Serum Creatinine (mg/dL)	0.95 ± 0.4	0.66 ± 0.32	> 0.05
Plasma total Homocysteine (μmol/L)	22.41 ± 7.78	13.2 ± 4.4	*< 0.001*
VOC/year	4.11 ± 2.9	NIL	NA
BT/year	1.42 ± 1.5	NIL	NA
Hospitalization/year	1.7 ± 1.5	NIL	NA

Signigicant *p*-value have been italics

ALT: alanine transaminase; *AST*: aspartate transaminase; *BT*: blood transfusion; *Hb*: hemoglobin; *HbF*: fetal hemoglobin; *HbS*: sickle hemoglobin; *HCT*: hematocrit; *LDH*: lactate dehydrogenase; *MCH*: mean corpuscular hemoglobin; *MCHC*: mean corpuscular haemoglobin concentration; *MCV*: mean corpuscular volume; *RBC*: red blood cell; *VOC*: vaso-occlusive crisis; *WBC*: white blood cell; *NA*: Not Available

institution, the SCA patients had higher mean episodes of mild VOC (15 ± 4.21/Year), followed by moderate (4.18 ± 2.4/year) and severe VOC (1.2 ± 1/year). Overall mean episodes of VOC was 4.11 ± 2.98/year. Interestingly, we found that patients had reported significantly more mean episodes of moderate VOC compared to that of severe VOC per year ($p < 0.001$). The incidence of blood transfusion (BT) among the SCA patients was 1.42 ± 1.5/year, and the episode of hospitalization was 1.7 ± 1.5/year (Table 1).

The clinical observation of the patients with SCA at the time of presentation showed that the common causes of presentation were anemia (89%) and moderate to severe VOC (80%), followed by hemolytic jaundice (35.84%), splenomegaly (28.33%), hepatomegaly (29.16%), cholelithiasis (13.33%), avascular necrosis (10.83%), and rarely due to ischemic stroke (4.16%) (supplementary TABLE-5).

The distributions of 120 SCA cases on references to mild VOC, moderate VOC, and severe VOC were 24 (20%), 57 (47.5%), and 39 (32.5%), respectively. The result obtained from the Turkey-Kramer multiple comparison tests of hematological and biochemical parameters within the three categories of VOC demonstrated a significant trend of difference. In both the mild, moderate, and severe VOC categories, Hcy and LDH showed a significantly high difference ($p < 0.001$) between categories with an increasing trend, while HbF% had decreased significantly between categories ($p < 0.001$). Other important parameters showing significant difference between the categories of VOC were Hb ($p < 0.01$), platelet ($p < 0.05$), Bil-D ($p < 0.05$), Bil-T ($p < 0.05$), and AST ($p < 0.01$) between mild and severe VOC categories and Bil-D ($p < 0.01$) and AST ($p < 0.05$) between moderate and severe categories. ANOVA analysis revealed a significant difference ($p < 0.05$) in between the

Table 2 Turkey-Kramer multiple comparison test and ANOVA among mild, moderate, and severe VOC of SCA of Odisha, India

	Mild VOC Mean ± SD	Moderate VOC	Severe VOC	Mild VOC vs Mod VOC *p*– value	Mild VOC vs severe VOC	Mod VOC vs severe VOC	ANOVA
HbF (%)	22.95 ± 5.3	20.84 ± 4.66	17.78 ± 3.8	> 0.05	*< 0.001*	*< 0.001*	*< 0.001*
HbS(%)	71.91 ± 4.5	73.32 ± 4.7	74.84 ± 5.4	> 0.05	> 0.05	> 0.05	0.07
WBC ($\times 10^3/\mu L$)	9.6 ± 3.6	9.8 ± 3.1	10.6 ± 4.5	> 0.05	> 0.05	> 0.05	0.45
RBC($\times 10^6/\mu L$)	3.1 ± 0.7	3.2 ± 0.69	2.9 ± .9	> 0.05	> 0.05	> 0.05	0.73
Hb (g/dL)	9.1 ± 1.4	8.4 ± 1.6	7.89 ± 1.3	> 0.05	*< 0.01*	> 0.05	*0.0081*
HCT (%)	25.6 ± 6	27.2 ± 7.57	25.27 ± 4.89	> 0.05	> 0.05	> 0.05	0.313
MCV (fL)	81.83 ± 10.3	85.44 ± 12.14	81.83 ± 10.37	> 0.05	> 0.05	> 0.05	0.32
MCH (pg)	26.65 ± 4	27.69 ± 3.39	26.65 ± 3.2	> 0.05	> 0.05	> 0.05	0.26
MCHC (g/dL)	31.54 ± 4.6	32.54 ± 3.8	32.68 ± 3.6	> 0.05	> 0.05	> 0.05	0.49
Platelets ($\times 10^5/\mu L$)	3.21 ± 2	2.72 ± 1.54	2.16 ± 1.02	> 0.05	*< 0.05*	> 0.05	*0.048*
Bil-T (mg/dL)	1.98 ± 0.99	2.56 ± 1.56	3.15 ± 1.7	> 0.05	*< 0.05*	> 0.05	*0.0049*
Bil-D (mg/dL)	0.8 ± 0.4	0.79 ± 0.57	1.21 ± 0.8	> 0.05	*< 0.05*	< 0.01	*0.0045*
AST (U/L)	49.5 ± 16.22	55.21 ± 25.6	67.43 ± 15.1	> 0.05	*< 0.01*	*< 0.05*	*0.0023*
ALT (U/L)	52.7 ± 19.04	54.05 ± 24.8	63.41 ± 17.8	> 0.05	> 0.05	> 0.05	0.07
Serum Creatinine (mg/dL)	0.95 ± 0.52	0.96 ± 0.35	0.97 ± 0.36	> 0.05	> 0.05	> 0.05	0.95
LDH (U/L)	371.75 ± 125.72	747.24 ± 494.56	939.53 ± 262.04	*< 0.01*	*< 0.001*	> 0.05	*< 0.001*
Plasma total Homocysteine (μmol/L)	13.08 ± 2.3	21.85 ± 6.5	29.25 ± 9.8	*< 0.001*	*< 0.001*	*< 0.001*	*0.0002*

Signigicant *p*-value have been italics

ALT: alanine transaminase; *AST*: aspartate transaminase; *BT*: blood transfusion; *Hb*: hemoglobin; *HbF*: fetal hemoglobin; *HbS*: sickle hemoglobin; *HCT*: hematocrit; *LDH*: lactate dehydrogenase; *MCH*: mean corpuscular hemoglobin; *MCHC*: mean corpuscular haemoglobin concentration; *MCV*: mean corpuscular volume; *RBC*: red blood cell; *VOC*: vaso-occlusive crisis; *WBC*: white blood cell; *NA*: Not Available

categories of VOC for the variables, i.e., HbF%, Hb, platelet, Bil-T, Bil-D, AST, LDH, and Hcy levels (Table 2).

The result obtained from the photometric analysis showed that Hcy level was significantly higher ($p < 0.0001$) in patients with SCA (22.41 ± 7.8 μmol/L) compared to that of the controls (13.2 ± 4.4 μmol/L) (Table 1). Among the 120 SCA cases studied, 25.8% of cases were having normal Hcy level of < 15 μmol/L, 57.5% were in having moderate (15–30 μmol/L), while only 16.7% were having intermediate Hcy level (30–100 μmol/L). Among the SCA cases with normal Hcy level, none had reported an episode of severe VOC, while few SCA cases with moderate and mild VOC were found (9 and 22 respectively). However, all the SCA cases having severe VOC episodes had an either moderate or intermediate level of Hcy. A total of 39 SCA cases had reported with severe VOC, of which 25 had moderate Hcy level, and the remaining 14 had intermediate Hcy level. Interestingly, maximum SCA cases with moderate VOC (42/57) had moderate Hcy level. We found no SCA cases with severe Hcy level (> 100 μmol/L). It was also observed that elevated serum Hcy was more in female SCA patients (55.2% of the 58) than that in male patients (38.7% of 62), although the difference was not significant ($p > 0.05$).

Out of 120 SCA patients, 92 patients (76.7%) were on folic acid therapy (5 mg/day) as per prescription, and the remaining 28 did not receive the therapy. Patients without FA therapy showed statistically significant ($p < 0.001$) high Hcy levels (27 ± 7, median 28.1 μmol/L) compared to those who were on FA therapy (17.75 ± 5.7, median 16.9 μmol/L) and normal controls (13.2 ± 4.3, median 13 μmol/L; $p < 0.001$). A significant difference in Hcy levels ($p < 0.05$) was also found between normal controls and SCA patients on FA therapy (Fig. 1; Table 1).

Multiple regression analysis for the association of hematological, biochemical, and the different clinical presentations with serum Hcy level revealed a significant association ($p < 0.05$) with %Hb, LDH, FA, VOC, and stroke. The Pearson correlation analysis revealed that the serum Hcy level was positively correlated with AST ($r = 0.226$, $p = 0.014$), Bil-I ($r = 0.232$, $p = 0.011$), Bil-T ($r = 0.203$, $p = 0.027$), LDH ($r = 0.291$, $p = 0.001$), jaundice ($r = 0.205$, $p = 0.026$), stroke ($r = 0.579$, $p = 0.001$), VOC per year ($r = 0.449$, $p = 0.001$), and hospitalization per year ($r = 0.247$, $p = 0.007$) while negative correlation was observed with HbF% ($r = -0.232$, $p = 0.011$), Hb level ($r = -0.232$, $p = 0.001$), and patients on FA therapy ($r = -0.511$, $p = 0.001$) (Table 3).

The observed genotype and the allele frequency of MTHFR C677T gene polymorphism were in Hardy-Weinberg equilibrium (χ^2 test p value = 0.59) in the control group. The minor allele frequency (T) distribution among the SCA was calculated to be 0.1 and that in the control group was 0.07. There was no statistically significant difference between

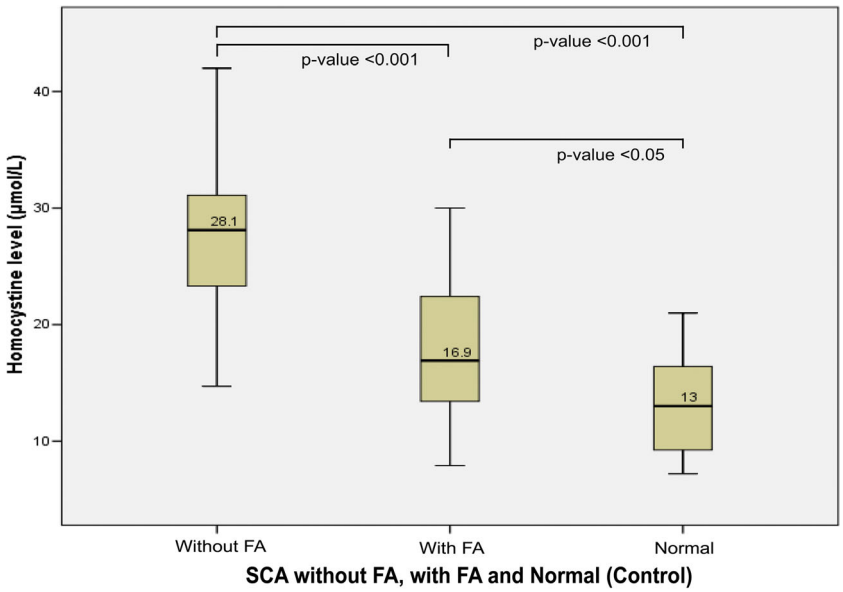

Fig. 1 Box plot: showing homocysteine level in SCA with, without folic acid supplement and normal

the genotypes (Odd Ratio (95% CI) = 0.68 (0.28–1.63); p = 0.53) (supplementary TABLE-4). Similarly, we did not find any apparent significant association ($p > 0.05$) between MTHFR C677T gene polymorphisms and clinical presentation in patients with SCA (supplementary TABLE-5). The mean Hcy levels of the three polymorphisms were 21.28 ± 7.4 μmol/L for CC, 24.86 ± 12.85 μmol/L for CT, and 47.5 ± 16.12 μmol/L for TT. ANOVA test revealed a significant difference ($p < 0.05$) in the Hcy level between CC, CT and TT genotypes.

Discussion

Among the pleiotropic effect of SCA, VOC has been reported as the most common clinical presentation in the earlier studies as well as in the present study [6, 40]. Although significantly higher Hcy level ($p < 0.0001$) in patients with SCA has been reported as compared to that in the normal controls [11, 13, 16, 17], it is not well documented whether the higher Hcy level leads to the severity of VOC. In the present study, we attempted to compare the level of Hcy between SCA and healthy control as well as against various VOC categories. We found that in the SCA patients, the mean level of Hcy was almost double the normal range. Furthermore, about three-fourth (7.4 in every tenth patient) of the SCA patients had an elevated level of Hcy while only one-fourth of the normal controls had a higher Hcy level.

The variation of Hcy has been attributable to the patterns of dietary varieties [41]. Brattstrom et al. (1988) reported that the oral supplementation of folic acid lowers the plasma Hcy level [42]. To evaluate the effect of folic acid supplementation, the patients in the present study were grouped as patients, not on FA therapy ($n = 28$) and patients with FA supplement ($n = 92$) with a dose of 5 mg/day. A significant difference at the Hcy level could be found between the two groups. Interestingly, higher Hcy level was recorded among the SCA patients not on FA therapy than among those on the therapy. However, we did not measure the FA level in RBC, plasma, or serum. The observation of a negative relationship between FA and Hcy ($r = -0.511$, $p = 0.001$; Table 3) is in agreement with the earlier studies [8–10]. This suggests that one of the mechanisms leading to folate deficiency in SCA patients is the higher demand due to chronic hemolysis, which determines a shorter lifespan of red cells and, consequently, a higher rate of erythrocyte production. However, studies did not show any correlation between the vitamins (B12, B6) and the level of Hcy among SCA patients [13]. Lowenthal et.al. (2000) found that FA is required by SCA patients for their better health status [16].

We found that in controls, the LDH and Hcy level were within the range, but in the SCA patients with an increase in severity of VOC, an increase in both LDH and Hcy level were recorded, indicating a possible effect of Hcy on the hemolysis of RBC. Our study demonstrates that increasing Hcy has a greater chance of hemolysis resulting in the increasing of LDH ($r = 0.291$, $p = 0.001$; Table 3). Earlier, an in vitro study by Lin et al. showed that increased Hcy induces cytotoxicity and cell lysis, marked by the release of LDH [43]. Many studies show that during severe VOC, there is an increase of LDH level [44–47]. These observations and our findings strongly suggest that among the SCA patients, Hcy may have a direct link with the severity of VOC through the hemolysis pathway process.

We infer from our results that in liver enzymes of AST, ALT, and total bilirubin, conjugated bilirubin was elevated during severe VOC as compared to mild VOC. This may be due to hyper hemolysis; there is an increase in liver enzyme activity to neutralizing heme toxicity. From correlation study, it is revealed that Hcy is directly related to the AST,

Table 3 Pearson correlation of homocysteine (dependent variable) with various parameters

	Pearson correlation (*r*)	*p* –value
CE-HPLC		
HbA	0.101	0.275
HbA2	0.152	0.099
HbS	0.089	0.334
HbF	*− 0.232**	0.011
Complete blood count		
WBC	0.01	0.918
RBC	−0.136	0.14
Hemoglobin	*− 0.295***	0.001
HCT	− 0.132	0.154
MCV	− 0.008	0.933
MCH	0.006	0.951
MCHC	0.013	0.885
Platelets	0.089	0.337
Biochemistry parameters		
AST	*0.226**	0.014
ALT	0.06	0.517
Bil-D	0.136	0.141
Bil-I	*0.232**	0.011
Bil-T	*0.203**	0.027
Creatinine	0.05	0.586
LDH	*0.291***	0.001
Clinical presentation		
Anemia	0.073	0.433
Jaundice	*0.205**	0.026
Splenomegaly	0.135	0.142
Hepatomegaly	0.097	0.295
Cholelithiasis	0.041	0.656
Avascular necrosis	− 0.042	0.649
Stroke	*0.579***	0.001
VOC/YR	*0.449***	0.001
BT/year	0.15	0.104
Hospitalization/year	*0.247***	0.007
Folic acid status	*− 0.511***	0.001

ALT: alanine transaminase; *AST*: aspartate transaminase; *Bil-D*: bilirubin-total; *Bil-I*: bilirubin-indirect; *Bil-T*: bilirubin-total; *BT*: blood transfusion; *Hb*: hemoglobin; *HbF*: fetal hemoglobin; *HbS*: sickle hemoglobin; *HCT*: hematocrit; *LDH*: lactate dehydrogenase; *MCH*: mean corpuscular hemoglobin; *MCHC*: mean corpuscular haemoglobin concentration; *MCV*: mean corpuscular volume; *RBC*: red blood cell; *VOC*: vaso-occlusive crisis; *WBC*: white blood cell;

*Correlation is significant at the 0.05 level (two-tailed)

**Correlation is significant at the 0.01 level (two-tailed)

unconjugated bilirubin, and total bilirubin but the conjugated bilirubin has no effect on the Hcy level. The observation of elevated liver enzyme values and bilirubin in SCA patients during crisis is in consonance to the study by Ojuwao et al. (1994) that this transit time of disturbance may be due to heptic cellular injury which may due to hemolysis [48].

In addition, we found that platelet count had decreased in severe VOC state compared to that in the mild state. It has been postulated that mhHcy plays a key role in thrombogenic state development by oxidative stress [49] and may lead to acute chest syndrome (ACS) as well as decrease in platelet count due to accumulation at the site of occlusion [50–54]. Our data suggest that about 60% of SCA patients studied were in mhHcy state reflecting a high possibility of being in the thrombogenic state.

Interestingly, high HbF% have a protective role in VOC as mild VOC have greater HbF% (22.95 ± 5.3) than severe VOC cases (17.78 ± 3.8), ($p < 0.001$), which is corroborated by previous study from this population [22, 23]. We found that SCA patients with higher HbF% (> 15%) tend to have a lower Hcy value and also had a lower frequency of moderate and severe VOC along with comparatively lower LDH level against SCA patients with lower HbF% (< 15%). Also in our study, Hcy was negatively correlated with HbF% ($r = -0.232$, $p = 0.011$). This suggests that the high HbF% benefits the SCA patients from severity of VOC to some extent. This study is the first to present the association of HbF with Hcy in literature.

A meta-analysis by Cronin et al. (2005) suggested that MTHFR 677 T allele is associated with ischemic stroke [55]. However, in our population, the minor allele frequency (MAF) was very low (10% in SCA and 7% in normal), and they were equally distributed in both cases and controls ($p > 0.05$). Lack of association of the minor allele with any of the clinical presentations of patients indicates that the major type allele (C) is widespread in the studied population. Only among the studied SCA patients with stroke, we found the mutant allele had higher Hcy levels (53.73 ± 6.2 μmol/L) than wild allele (39.5 ± 4.1 μmol/L). In the above context, some other polymorphisms of the MTHFR gene and cystathionine β synthase gene, involved in the Hcy metabolism pathway, might be interesting to study as they may reflect their effect on the level of Hcy in SCA patients in this population.

Conclusion

When the folic acid level is low, an environmental interaction such as food habit, adaptation, and life style may contribute to the vascular problem in SCA by increasing Hcy level. Folate supplementation is required on a regular basis for patients with SCA. Our study suggests that with an increase in Hcy level, hemolysis and platelets might have an influence on the VOC of patients with SCA in Odisha. HbF% in conjunction with lower Hcy level, on the other hand, seems to have an important role in lowering the frequency of moderate and severe VOC. The MAF of C677T MTHFR is relatively less common and is not associated with vascular complicacy in our

population, and other enzyme polymorphisms may be involved in the folic acid metabolism pathway for lowering the Hcy level which lay the open path for further studies.

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Author contributions Conceptualization and design: SM, BPD, and PKM. Data collection: SM, SP, KD, SD, BPJ, MMM. Laboratory work: SM. Data analysis and interpretation: SM, SP, PD, BPD, PKM. Manuscript writing: all authors. Final approval of manuscript: all authors.

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Compliance with ethical standards

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional ethical committee of VIMSAR, Burla, Odisha (VIREC-No.2016/I-F-CT-01/008) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed written consent was obtained from all individual participants included in the study.

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Conflict of interest The authors declare that they have no conflict of interest.

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