Interactions of Combined Haplotypes of Methylenetetrahydrofolate Reductase on Clinical Events in Children with Sickle Cell Disorder

Suprava Patel¹, Rachita Nanda², Nighat Hussain³, Eli Mohapatra⁴, Pradeep Kumar Patra⁵

^{1,2,4}Department of Biochemistry, All India Institute of Medical Sciences, Raipur, Chhattisgarh, India, ³ Department of Pathology, All India Institute of Medical Sciences, Raipur, Chhattisgarh, India, ⁵ Department of Biochemistry, Pt. Jawaharlal Nehru Memorial Medical College, Raipur, Chhattisgarh, India.

ABSTRACT

BACKGROUND

Methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C variants are considered as potential genetic risk factors for vaso-occlusive complications in sickle cell disorder (SCD). The purpose of the study was to determine the interaction of the combined haplotypes on the clinical presentations in children with sickle cell disorder.

METHODS

A cross-sectional study was conducted on 249 children, confirmed for sickle cell disorder. Clinical details and frequencies of clinical episodes in the past one year were noted and a severity index number was allotted to each child and evaluated for their relationship with the combined haplotypes of C677T and A1298C single nucleotide polymorphisms genotyped by real-time PCR.

RESULTS

The frequency for 677T / 1298A haplotype was 46.4 % and that of 677C / 1298C was 12.4 %. The three variant combined haplotypes had higher plasma homocysteine values than the wild 677C / 1298A haplotypes ($P < 0.001$). Clinical events like vaso-occlusive crisis (VOC), homocysteinemia, hospitalization frequency and SI were found significantly related among the children in sickle cell trait (SCT) group ($P < 0.001$) but not so in SCD group. Chances for anemia was 1.93 times more in presence of dual variant alleles (95 %CI: 0.95 to 3.92, $P = 0.07$) in SCT. The 677T / 1298C haplotype accounted for higher SI was 7.85 times more than the wild haplotypic children even in SCT children and 1.67 times in SCD children.

CONCLUSIONS

Presence of the variant haplotypes had significant implication on crisis events in children with sickle cell trait and make them more prone for the clinical severity. A preliminary allelic screening might be helpful in them.

KEY WORDS

Dual Variant Alleles, Heterozygous, Homozygous, MTHFR, Variant Haplotypes

Corresponding Author:

Dr. Suprava Patel,
Department of Biochemistry,
All India Institute of Medical
Sciences, Raipur,
Chhattisgarh, India.
E-mail: dr_suprava@yahoo.co.in

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BACKGROUND

Sickle cell anemia (SCA) is of major public health importance in this region of Central India and is known by its perplexing aspect of varied clinical manifestations. Homocysteinemia induced endothelial dysfunction, platelet activation, activated coagulation cascade are the mainstay pathophysiologies to create a hypercoagulable state resulting in vaso-occlusive phenomenon which is frequently observed in this disorder.¹⁻³ Besides nutritional factors like folic acid or vitamin B12 deficiency, recent studies reported that single nucleotide polymorphisms (SNPs) in enzymes involved in methionine - homocysteine metabolism as in methylenetetrahydrofolate reductase (MTHFR) enzyme which maintains the active folate level can lead to homocysteinemia.^{3,4} Presence of C677T and A1298C SNPs of MTHFR enzyme in SCA have epistatic effects on the clinical presentation in these cases.^{1,3,5} Co-existence of these SNPs with sickle cell gene can increase the homocysteine levels and thus are regarded as potential genetic risk factors for vaso-occlusive or hemolytic episodes with acute painful events in them.⁶⁻⁸ However, very few studies are available regarding the contributory and additive effect of both the polymorphisms towards the clinical complications witnessed in SCA patients in pediatric age group who are most vulnerable for the varied manifestations of the disorder and register a high morbidity and mortality.^{9,10} Taking into account the above facts, the study was intended primarily to determine the interaction of the combined haplotypes of MTHFR SNPs on the clinical presentations in children diagnosed with sickle cell disorder. To the best of our knowledge, the present study is first of its kind in this region to determine their role as potential genetic risk factors for the vaso-occlusive crisis in them.

METHODS

The cross-sectional study was conducted after approval from the Institute Ethical Committee. Children of age group 5 to 18 years diagnosed with sickle cell disorder, attending our institute were included for the study. The prevalence of MTHFR SNP ranged from 15 – 19.3 %. Rounding off to 20 % prevalence and using Cochran's formula, $N = Zpq / e^2$, the sample size was calculated to be 384, rounded off to be 350. But the sample size target could not be achieved and only 249 cases could be enrolled during the study period from March 2017 to March 2019. Total 249 children were enrolled after written informed consent from their parents / legally accepted guardians by convenient sampling method. The study population comprised of 218 HbAS (heterozygous) and 31 HbSS (homozygous) cases as per their high performance liquid chromatography (HPLC) based confirmatory report. The former were grouped as sickle cell trait (SCT) in children and the later as sickle cell disease (SCD). Demographic and clinical details were entered in pre-structured questionnaire used in our institute. Clinical history of past one year was noted for all the children for frequencies of vaso-occlusive crisis (VOC) or acute hemolytic crisis, acute painful events, infective episodes, blood transfusions and hospitalization. Severity Index (SI) number was allotted to each child by summing up the number of past and present events as described above. The children were subcategorized as those with a SI of ≤ 6 and those with > 6 for comparison among the study groups. Blood hemoglobin

and plasma homocysteine were evaluated after enrollment to know the status of anemia and homocysteinemia.

Once enrolled, 3 ml of blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainer and transported to laboratory in chiller box. Hemoglobin (Hb) was measured in XP - 100 Fully Automated hematology Analyzer, Sysmex. 500µl blood was aliquoted and stored at 4° to 8°C for deoxyribonucleic acid (DNA) extraction using Invitrogen™ PureLink™ Genomic DNA Mini kit from ThermoFisher Scientific on weekly basis. Extracted DNA was stored at - 80°C until processed for real-time polymerase chain reaction (PCR). Remaining blood was centrifuged for plasma separation which was aliquoted and stored in - 80°C to be analyzed later for plasma homocysteine (Hcy) in Advia Centaur XP Fully Automated Chemiluminescence Immunoanalyzer, Seimens.

The MTHFR polymorphisms under study were genotyped for C677T (rs1801133) and A1298C (rs1801131) (Assay ID's C_1202883_20 and C_850486_20 respectively) using TaqMan SNP Genotyping PCR Kit from Applied Biosystems, ThermoFisher. PCR was processed in CFX96 real time PCR system, Biorad. The C677 allele of C677T was considered as the wild allele and T677 allele as the variant allele whereas for A1298C, allele A1298 was considered as wild and allele C1298 as the variant allele. As both SNPs has C alleles, to avoid the confusion for the readers, the variant allele of A1298C will be presented as C' and the wild allele of C677T as C in the manuscript henceforth.

Combined haplotypes, 677C / 1298A, 677C' / 1298C', 677T / 1298A and 677T' / 1298C', each comprising of either of the alleles of the MTHFR variants were evaluated for their combined effect on the clinical variables. 677C / 1298A consisting of both the wild alleles was considered as wild haplotype. The other haplotypes comprising of at least one variant allele of either SNPs were considered as variant haplotypes. 677T / 1298C' combined haplotype with both variant alleles was noted as the dual variant haplotype. The allelic and haplotypic frequency percentages were calculated from the total allelic population which was considered as $N * 2$ ($249 * 2$) = 498.

Children with blood Hb level ≤ 11 gm / dL were considered under clinical variables for anemia.^{11,12} The cut off value for plasma Hcy was 13µmol / L (as per kit insert) beyond which it was considered homocysteinemia.

Ethical Statement

This research was reviewed and approved by the Institutional Ethical Committee of All India Institute of Medical Sciences, Raipur, Chhattisgarh, India. Informed consent was obtained from all participants.

Statistical Analysis

All statistical calculations were computed in IBM®SPSS version 20. Haplotypic frequency was presented as percentage and the quantitative variables were presented as mean with standard deviation (SD). Chi-Square test was applied for distribution of the combined haplotypes among the groups. Comparison of the quantitative variables among the haplotypes in the study population was extrapolated using independent t - test. Further analysis by logistic regression along with odd ratio (OR) and 95 % confidence interval (CI) was performed to predict the relationship between the haplotypic variants with the clinical outcomes. P - value < 0.05

was accepted for statistical significance in this study. Allelic population of 498 was considered for performing the statistical analysis. For all comparisons, the wild haplotypic (677C / 1298A) children were considered as the reference category.

RESULTS

Characteristics of the Study Population and the Haplotypic Distribution

The study population consisted 87.6 % (N = 218) SCT and 12.4 % (N = 31) SCD children of 5 to 18 years age group. The mean (SD) age of the children was 11.27 (3.47) years and comprised of 137 (13.8 %) female and 112 (11.2 %) male children. The overall frequency percentages of clinical events, including both present and past history, in the study population are depicted in Figure - 1. Homocysteinemia and anemia were prevalent with 71.9 % and 65.5 % of the allelic population at the time of enrollment. The episodes of VOC, painful events, infections and hospitalization ranged from 20 % to 40 %. Only 4 % of these children gave history of blood transfusions. 11.2 % children of the total allelic population documented SI > 6.

The distribution of the combined haplotypes in the study population outlined in Table 1, was merely different between the study groups (P = 0.05). The frequency percentages of variant alleles T677 and C'1298 were 15.1 % and 44.4 % respectively. The odds for dual variant haplotype 677T / 1298C' was 2.52 times (95 %CI: 1.13 to 5.5, P = 0.008) in the SCD group when compared to the reference haplotype.

Comparison of Quantitative Variables within the Haplotypic Variants

The comparison of the quantitative variables among the four haplotypes is illustrated in Figure 2. Children with 677T / 1298C' haplotype documented significantly lower body mass index (BMI) (P = 0.015) and Hb levels (P = 0.006) when compared to children with 677C / 1298A haplotype. The three variant combined haplotypes had higher plasma homocysteine values than the wild haplotypes (P < 0.001).

Regression Analysis of Combined Haplotypes and the Clinical Variables in SCT Children

Inter - relationship of MTHFR combined haplotypes with the clinical events in SCT group was analyzed using logistic regression analysis and is delineated in Table 2. Clinical events like VOC, infective episodes, homocysteinemia, hospitalization frequency and SI were found significantly related among the groups (P < 0.001). 25.5 % of SCT children who documented history of frequent VOC events, were reported for 677T / 1298C' in SNP genotyping as against only 7.4 % with no VOC events.

The odds for vascular crisis in dual variant haplotypes was 3.79 times (CI 95 %: 1.99 to 7.24, P < 0.001) more than the

reference category in SCT children. Co-existence of dual variant alleles raised the chances for painful episodes by 59 % and anemia by 1.93 times. The prediction for severe grade anemia (Hb < 7 gm / dl) was found to be increased by 42 % in SCT children (95 % CI: 0.29 to 5.48, P = 0.61) in these haplotypes (data not shown).

Similarly, the variant haplotypes revealed significant relationship with homocysteinemia and hospitalization frequency (P < 0.001). All children with 677T / 1298C' haplotype had homocysteinemia at the time of enrollment. The odds for frequent hospitalizations (> 2 times a year) was greatly influenced (49.79 times) in variants even though they are traits for the disease (data not shown). Similarly, the likelihood for frequent blood transfusions was increased by 42 % in C677T / 1298C'. Presence of dual variant alleles rendered these children towards higher SI score (7.85 times) than the reference haplotypes.

Regression Analysis of Combined Haplotypes and the Clinical Variables in SCD Children

As delineated in Table 3, the regression analysis of the MTHFR combined haplotypes depicted no significant relationship with the clinical events in SCD children. 100 % of the 677T / 1298C' (N = 12) haplotypes of this group depicted anemia and homocysteinemia at the time of enrollment. The odds for blood transfusion was 4.5 times (CI 95 %: 0.68 - 29.81, P = 0.12) in these haplotypes as against the reference group. The chances for a higher SI score were more by 67 % in children with dual variant alleles.

	SCT Allele N = 436 N (%) ^	SCD Allele N = 62 N (%) ^	χ^2_{df} , P - Value
677C / 1298A 231(46.4 %)	211(48.4)	20(32.3)	7.93 ₃ , 0.05
677C / 1298C' 127(25.5 %)	111(25.5)	16(25.8)	
677T / 1298A 78(15.7 %)	64(14.7)	14(22.6)	
677T / 1298C' 62(12.4 %)	50(11.5)	12(19.4)	
Table 1. Distribution of the Combined Haplotypes of C677T and A1298C MTHFR SNPs within the Study Population			
χ^2_{df} represents the chi square value with degree of freedom ^N (%) represents the column percentages

Figure 2. Comparison of Mean(SD) of Quantitative Variables among the Four Combined Haplotypes of MTHFR (N = 498 and df = 497)

*Significant at P < 0.05 when compared to the wild combined haplotype 677C / 1298A

	Presence of Clinical Events	677C / 1298A ^R (211)	677C / 1298C' (111)	677T / 1298A (64)	677T / 1298C' (50)	χ^2_{df} , p - Value
	N (%)	N (%)	N (%)	N (%)	N (%)	
VOC	98 (22.5)	44 (44.9)	18 (18.4)	11 (11.2)	25 (25.5)	22.16 ₃ , < 0.001*
	OR (CI 95 %)	Reference	0.74 (0.4 - 1.3)	0.79 (0.38 - 1.63)	3.79 (1.99 - 7.24)	
APE	66 (15.1)	35 (53)	11 (16.7)	8 (12.1)	12 (18.2)	6.02 ₃ , 0.11
	OR (CI 95 %)	Reference	0.55 (0.27 - 1.14)	0.72 (0.32 - 1.64)	1.59 (0.76 - 3.34)	
IE	154 (35.3)	88 (57.1)	40 (26)	7 (4.5)	19 (12.3)	23.91 ₃ , < 0.001*
	OR (CI 95 %)	Reference	0.79 (0.49 - 1.27)	0.17 (0.07 - 0.39)	0.86 (0.46 - 1.61)	
Anemia	272 (62.4)	131 (48.2)	67 (24.6)	36 (13.2)	38 (14)	5.18 ₃ , 0.16
	OR (CI 95 %)	Reference	0.93 (0.58 - 1.49)	0.78 (0.45 - 1.38)	1.93 (0.95 - 3.92)	
Hcynemia	308 (70.6)	109 (35.4)	87 (28.2)	62 (20.1)	50 (16.2)	101.87 ₃ , < 0.001*
	OR (CI 95 %)	Reference	3.39 (2.0 - 5.74)*	29 (6.92 - 121.64)*	---	
BT	10 (2.3)	6 (60)	0 (0)	2 (20)	2 (20)	6.13 ₃ , 0.11
	OR (CI 95 %)	Reference	---	1.1 (0.22 - 5.59)	1.42 (0.28 - 7.27)	
Hosp.	102 (23.4)	39 (38.2)	19 (18.6)	13 (12.7)	31 (30.4)	39.75 ₃ , < 0.001*
	OR (CI 95 %)	Reference	0.91 (0.49 - 1.67)	1.12 (0.56 - 2.27)	7.19 (3.69 - 14.04)*	
SI	> 6 38(8.7)	13 (34.2)	1 (2.6)	7 (18.4)	17 (44.7)	40.69 ₃ , < 0.001*
	OR (CI 95 %)	Reference	0.14 (0.02 - 1.07)	1.87 (0.71 - 4.91)	7.85 (3.49 - 17.65)*	

Table 2. Regression Analysis Showing the Relationship of the Presence of Clinical Events in SCT Cases with the MTHFR Combined Haplotypes (Considering the Wild Haplotype 677C / 1298A as Reference Group; N = 436)

χ^2_{df} represents the chi-square (Likelihood ratio tests) value with degree of freedom for Present VS Absent of events within the four groups; N (%): represents the row percentages; OR(CI 95 %): odds ratio with 95 % confidence interval; *represents significance level at P < 0.001; *represents wild haplotype 677C / 1298A was considered as the reference category for comparison; VOC: vaso occlusive crisis events; APE: acute painful events; IE: infective episodes; Hcynemia: homocysteinemia; BT: blood transfusions; Hosp.: Hospitalizations; SI: severity index; SI ≤ 6 was considered as reference category; could not be calculated because of very less number

	Presence of Clinical Events	677C / 1298A ^R (20)	677C / 1298C' (16)	677T / 1298A (14)	677T / 1298C' (12)	χ^2_{df} , p - Value
	N (%)	N (%)	N (%)	N (%)	N (%)	
VOC	58 (93.5)	19 (32.8)	15 (25.9)	13 (22.4)	11 (19)	0.15 ₃ , 0.98
	OR (CI 95 %)	Reference	0.79 (0.05 - 13.69)	0.68 (0.04 - 11.95)	0.58 (0.03 - 10.21)	
APE	52 (83.9)	17 (32.7)	13 (25)	12 (23.1)	10 (19.2)	0.14 ₃ , 0.99
	OR (CI 95 %)	Reference	0.77 (0.13 - 4.43)	1.06 (0.15 - 7.34)	0.88 (0.13 - 6.22)	
IE	52 (83.9)	16 (30.8)	14 (26.9)	14 (26.9)	8 (15.4)	7.44 ₃ , 0.06
	OR (CI 95 %)	Reference	1.75 (0.28 - 11.05)	---	0.5 (0.09 - 2.54)	
Anemia	54 (87.1)	17 (31.5)	12 (22.2)	13 (24.1)	12 (22.2)	3.85 ₃ , 0.28
	OR (CI 95 %)	Reference	0.77 (0.13 - 4.43)	1.06 (0.15 - 7.34)	---	
Hcynemia	50 (80.6)	14 (28)	12 (24)	12 (24)	12 (24)	7.01 ₃ , 0.07
	OR (CI 95 %)	Reference	1.29 (0.29 - 5.66)	2.57 (0.44 - 15.19)	---	
BT	10 (16.1)	2 (20)	2 (20)	2 (20)	4 (40)	2.96 ₃ , 0.39
	OR (CI 95 %)	Reference	1.29 (0.16 - 10.29)	1.5 (0.19 - 12.15)	4.5 (0.68 - 29.81)	
Hosp.	56 (90.3)	16 (28.6)	14 (25)	14 (25)	12 (21.4)	7.35 ₃ , 0.06
	OR (CI 95 %)	Reference	1.75 (0.28 - 11.05)	---	---	
SI	≤ 6 ^R	14 (31.8)	12 (27.3)	11 (25)	7 (15.9)	1.42 ₃ , 0.7
	> 6 18(30.6)	6 (33.3)	4 (22.2)	3 (16.7)	5 (27.8)	
	OR (CI 95 %)	Reference	0.78 (0.18 - 3.42)	0.64 (0.13 - 3.14)	1.67 (0.37 - 7.24)	

Table 3. Regression Analysis Showing the Relationship of MTHFR Combined Haplotypes with the Clinical Events in SCD Cases (N = 62)

χ^2_{df} represents the chi - square (Likelihood ratio tests) value with degree of freedom for Present vs Absent of events within the four groups; N (%): represents the row percentages; OR(CI 95 %): odds ratio with 95 % confidence interval; *represents significance level at P < 0.001; *represents wild haplotype 677C / 1298A was considered as the reference category for comparison; VOC: vaso-occlusive crisis events; APE: acute painful events; IE: infective episodes; Hcynemia: homocysteinemia; BT: blood transfusions; Hosp.: Hospitalizations; SI: severity index; SI ≤ 6 was considered as reference category; ---: could not be calculated because of very less number.

DISCUSSION

Vascular complications greatly influence the morbidity in children with sickle cell disorder even after regular folic acid supplementation, especially in homozygous SCD cases. Coexistence of any other polymorphism, like MTHFR polymorphism that impairs folic acid utilization leading to homocysteinemia would further complicate the scenario, even in heterozygous subjects. Considering the facts, the study was planned to evaluate the impact of the MTHFR haplotypes on the clinical outcomes in children with sickle cell disorder. The cross-sectional study included 249 children with confirmed

diagnosis of sickle cell disorder, out of which 87.6 % were heterozygous SCT and 12.4 % were homozygous SCD.

Few studies in various geographical locations sketched varied prevalence data for each of the variant alleles, but not much for the dual polymorphisms to compare with. The T677 allele frequency varied from 57.3 % in Central India, 0.03 % in Western India and 13 % in North India.^{5,13,14} The overall T677 allele frequency was 15.1 % in our study population which was comparable to Pandey et al.⁵ The frequency of C'1298 was reported 15 % in Lagos, Nigeria and 40.9 % in Brazilian population.^{3,15} Present study recorded 44.4 % prevalence of C'1298. Interaction of genetic and environmental factor might play a role in the varied distribution of these polymorphisms

in different regions. The distribution of the four combined haplotype between the groups did show some significant association ($P = 0.05$) depicting higher prevalence of 677T / 1298C' in SCD group (19.4 %) than SCT cases (11.5 %) (Table 1). The overall prevalence of dual variant haplotypes was 12.4 % that accorded to the noted prevalence of 9.1 % to 15.2 % in Hatzlhofer et al. study in 277 HbSS cases.¹⁵

Presence of the MTHFR variants also highly influenced the rise in plasma homocysteine level and frequency of hospitalization ($P < 0.001$). Various articles have reflected that repeated events of vaso-occlusive phenomenon leading to frequent hemolytic and painful crisis result in frequent hospitalization. These events often perpetuate towards higher incidences of morbidity like impaired growth, anemia in pediatric age group that also explains the lower BMI ($P = 0.015$) and blood hemoglobin ($P = 0.006$) in these children (Figure - 2).^{10,16,17} Homocystenemia is often focused in clinical conditions associated with vascular and thrombotic events which is considered a common phenomenon in homozygous SCD cases.^{4,18-20} However, presence of both the variant alleles, T677 and C'1298 documented significant influence on VOC, homocystenemia and frequency of hospitalization in SCT group (Table - 2). Hatzlhofer et al. study reported 15.2 % prevalence of double mutant 677T / 1298C' haplotype in VOC cases as against 9.1 % in cases without vascular events ($P = 0.192$) and predicted T allele as a potential risk allele for vascular complications in HbSS cases ($P = 0.015$) when compared to C' allele ($P = 0.913$).¹⁵ Similarly, other studies also predicted T allele as a risk factor for vaso-occlusive phenomenon seen in SCD cases.^{1,13,21} Similar to these studies, our study indicated that coexistence of dual mutant alleles in children significantly increased the risk of VOC by 3.79 times than the wild group (Table - 2) even in trait group. On further analysis, it was observed that 50 % of the children who documented vascular crisis more than two times in that year were 677T / 1298C' haplotypes (data not shown). The resultant effect of frequent vascular complications might have implicated for higher frequency of painful crisis (by 59 %), hospitalization (7.19 times, $P < 0.001$) and blood transfusions (1.42 times, $P = 0.67$) observed in SCT children. Eventually these children tend to have higher severity index (7.85 times) than the wild haplotypes (Table - 2) although the combined haplotypes failed to reveal significant relationship with the clinical events in SCD group.

The dual variant haplotypes were more prone for blood transfusions and 100 % of them documented severe grade of anemia, homocystenemia and frequent hospitalization (Table - 3). This could be attributed to the fact that the said mutations reduce the enzyme activity to 30 % and 70 % for homozygous C'1298 and T677 respectively.^{22,23} Thus, presence of dual variant alleles might have additive effect for reduced enzyme activity leading to significant homocystenemia. Further studies in the variant haplotypes might add valuable insight regarding the additive effects of reduced enzyme activity. Raised plasma homocysteine is implicated for endothelial dysfunction and an impending cause for thrombosis leading to vaso-occlusive events.^{2,3,8,18} Hence, presence of variant alleles predisposes for higher SI in children ascertained by summing up of the frequency of the clinical events.^{11,24} In agreement to the fact, both the SCT as well as SCD children depicted higher probability of SI > 6 in 677T / 1298C' haplotypes (Table 2 & 3). In spite of folic acid supplementation in these children, elevated plasma homocysteine indicated that the dose needs to be modified to meet the demand of folic acid and vitamin

B12 in children with mutant haplotypes of C677T / 1298C' or may be active folate, L - methyl folate that needs to be added.²⁵⁻²⁷ Few studies documented raised homocysteine levels in SCA cases in spite of FA supplementation and explained significant contributory effect of variant allele with B12 deficiency and homocystenemia ($P < 0.001$).²⁸⁻³⁰ Present study reflected implication of dual variant alleles on anemia with an odds of 2.34 times (CI 95 %: 1.18 - 4.64, $P = 0.015$) in the study population (data not shown). Various other studies also related MTHFR mutation to anemia and thus predicted these genetic polymorphisms towards abnormal indices pertaining to megaloblastic anemia which is again not uncommon in children with sickle cell disorder even after folate supplementation.³⁰⁻³²

CONCLUSIONS

Coexistence of both variant alleles of MTHFR C677T and A1298C' poses significant implication on vaso-occlusive events and frequency of hospitalization in children with SCA, especially in SCT children, that renders increased vulnerability towards higher severity index by 7 - 8 times. A preliminary allelic screening might be helpful in identifying the high risk children.

A substantial dose modification should be considered for these children with copresence of variant alleles. Accordingly, folic acid and vitamin B12 supplementations can be modified to control homocysteine levels and thus reduce the frequency of crisis events.

An active form of folate might be beneficial for these groups of children instead of regular folic acid supplementation. However, further large scale studies are still required to accurately evaluate the association of these two MTHFR polymorphisms with the complications of sickle cell disorder.

Study Limitations

The major limitations of this study reside in the fact that it was a hospital based study and quantitative evaluation of plasma folate and vitamin B12 was not undertaken due to fund constraint. Blood hemoglobin and plasma homocysteine reflected the levels at the time of admission as previous reports were found missing or not available in most cases at the time of enrollment. Further large scale studies in community level including the quantitative evaluation of blood parameters might provide more accurate information regarding the risk association. Quantification of these values in children without sickle cell disorder would have provided a more defined comparison of the plasma levels between cases and control. However, this study intended to determine the effect of SNPs on clinical variables, the severity index to be more precise, between the cases and not just mere comparison of the quantitative variables.

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