DOI: 10.7860/JCDR/2023/58342.17445

Original Article

Biochemistry Section

Association of β-Globin Gene Haplotypes with Haematological Parameters and Foetal Haemoglobin among Patients with Sickle Cell Disorder in Raipur, Chhattisgarh, India

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ABSTRACT

Introduction: Sickle cell disease is caused by a single nucleotide substitution in the β-globin gene. The variations in Foetal Haemoglobin (HbF) levels, β-globin gene cluster haplotype have been used as predictors of disease severity in sickle cell disease patients.

Aim: To determine the frequency of β-globin gene haplotypes in sickle cell disease patients and also to establish their association with haematological parameters and HbF.

Materials and Methods: The present cross-sectional study was conducted in Department of Biochemistry, Government Medical College, Jagdalpur, Chhattisgarh, India, in collaboration with Department of Biotechnology, Government Nagarjuna PG College of Science, Raipur, Chhattisgarh, India, from April 2021 to May 2022. A total of 100 patients with Sickle Cell Disease (SCD) and 50 with sickle cell traits were included in the study. Haplotypes were identified by Restriction Fragment Length Analysis (RFLP) method for seven polymorphic sites in β-globin gene cluster. Haematological parameters such as Hb, Haematocrit (HCT), Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH) and HbF levels were estimated. Data was analysed using various statistical tests such as Shapiro-Wilk test, Levene's test, student t-test, Mann-Whitney test and Kruskal-Wallis tests as per analysis requirement.

Results: In the present study, 51 (51%) males and 49 (49%) females were in sickle cell disease group (SS), while 25 (50%) males and 25 (50%) females were in sickle cell trait group (AS). The mean age of the sickle cell disease patients was 23.84±8.38 years and for sickle cell trait group was 26.3±7.37 years. There was a significant difference (p-value <0.0001) in HbF levels among haplotypes. Additionally, higher HbF concentration was found in Arab-Indian haplotypes in SCD patients. No significant association was observed between the haplotypes and haematological parameters.

Conclusion: The findings suggested that haematological parameters were not significantly associated with β-globin gene haplotypes. The β-globin gene haplotypes influence the HbF levels in sickle cell patients.

Keywords: Chromatography, Disease severity, Restriction fragment length analysis, Sickle cell traits

INTRODUCTION

Sickle Cell Disease (SCD) is caused by a single nucleotide substitution in the β-globin gene. However, despite being a monogenic disease, sickle cell disease has been associated with diverse range of disease severity. The severity of sickle cell disease has correlation with various clinical phenotypes between different individuals [1]. The clinical severity is influenced by variations in Foetal Haemoglobin (HbF) levels, the β-globin gene haplotype and presence of α-thalassaemia [2].

Foetal Haemoglobin is a well-known major genetic modulator of the clinical heterogeneity observed in sickle cell disease patient [3-5]. Higher HbF levels protect against many of the haematologic and clinical complications of sickle cell anaemia. Recently, it is reported that higher expression of HbF in adulthood ameliorates morbidity and mortality in sickle cell disease [6-9]. In this context, much work has been done for analysis of genetic determinants in the β gene cluster that might affect globin gene expression and thus relate to the clinical diversity of sickle cell disease [10-12]. This observation stimulated research to identify genetic determinants associated with disease severity such as β-haplotypes based on Restriction Fragment Length Analysis (RFLP) analysis in Chhattisgarh, India.

The β-globin gene haplotype is characterised by the non random association of cleavage sites recognised by restriction endonucleases enzymes [13]. Five major β-haplotypes designated as Benin, Bantu, Cameroon, Senegal and Arab-Indian haplotypes are named according to the geographic region or ethnic group in which they were most commonly found [14-18]. Other less common haplotypes, known as atypical haplotypes, are generated by a number of genetic mechanisms [19-21].

Till date, the relationship between β^S haplotypes and HbF level with haematological parameters has not been defined for SCD patients in Chhattisgarh. A better understanding of β-globin gene haplotypes is of particular relevance to population genetic studies and may also be useful for understanding the varying clinical outcomes seen among individuals with sickle cell disease. Hence; present study was conducted to determine the frequency of β^S haplotypes and their association with HbF level, haematological parameter in SCD patients of Chhattisgarh, India.

MATERIALS AND METHODS

This cross-sectional study was conducted in the Department of Biochemistry, Government Medical College Jagdalpur, India, in collaboration with Department of Biotechnology, Government Nagarjuna PG College of Science, Raipur, Chhattisgarh, India, from April 2021 to May 2022. The study was approved by the Institutional Ethical Committee (reference no.3572/GMCJ/Estt/22). Written informed consent was taken from all the subjects before commencement of the study.

Inclusion criteria: Patients with positive result of solubility test, cellulose acetate electrophoresis and Sickle Haemoglobin (HbS) mutation {confirmed using Polymerase Chain Reaction (PCR)-RFLP method of Dde I restriction enzymes}, were included in the study.

Exclusion criteria: Patients with systemic disease like Human Immunodeficiency Virus (HIV) and renal failure, taking any kind of treatment (other than folic acid supplementation) and blood transfusions during the four months prior to sample collection were excluded from the study. Pregnant women and patients with sickle cell disease and sickle cell trait belonging to same family were also excluded from the study.

Sample size calculation: A total of 100 sickle cell disease patients (SS) and 50 sickle cell traits (AS) were enrolled in the present study by convenience sampling. Samples were collected by random sampling method.

Demographic data such as age, gender were collected from all the participants. Venous blood samples (5 mL) were collected under aseptic precautions, using Ethylenediamine Tetraacetic Acid (EDTA) as an anticoagulant.

Molecular Diagnosis of Patients (PCR-RFLP Method)

Genomic Deoxyribonucleic Acid (DNA) was extracted using Himedia DNA kit and amplified by PCR (Bio-Rad thermal cyclers, model no. T100) using forward 5'-ACC TCA CCC TGT GGA GCC AC-3' and reverse 5'-GAG TGG ACA GAT CCA AAG GAC TCA AAG A-3' primers followed by restriction digestion and agarose gel inspection. The presence of an A or T nucleotide in the 6th codon of β-globin was confirmed for all DNA samples using the restriction enzyme Dde I analysis (fermentas) [22].

Polymerase chain reaction mix were prepared by adding the following:

- 2.5 μL of 10x buffer,
- 1.5 μL (25 mM) $MgCl_2$,
- 1.56 μL (2 Mm) deoxynucleoside triphosphate dNTP's,
- 1 μL of forward and reverse primer (10 picomoles),
- 0.5 μL of taq polymerase and nuclease free water 15.9 μL, and
- 1 μL of genomic DNA (5 ng/μL).

The reactions consisted of an initial step of DNA denaturation at 94°C for three minutes, followed by 35 cycles of one minutes at 94°C for denaturation, one minutes at 56°C for annealing, one minutes at 72°C for polymerisation and a final step of seven minutes at 72°C.

Haematological Study

Complete blood counts were carried out using an automated cell counter (BC-3000 Plus Auto Haematology Analyser). Haematological parameters such as Hb level, Haemotocrit (HCT), Mean Corpuscular Value (MCV), Mean Corpuscular Haemoglobin (MCH) were studied by haematological analysis [23].

HbF Level Determination

The HbF% was quantified by ion-exchange high-performance liquid chromatography. High Performance Liquid Chromatography (HPLC) was carried out using VARIANTTM β-thalassaemia Short Program, Bio-Red L 70018803 model instrument equipped with a dual-wavelength filter photometer (415 and 690 nm) [24].

Haplotype Analysis by RFLP

Polymerase chain reaction based restriction enzyme digestion method was used for haplotype analysis with following polymorphic restriction site; Hinc II ε, Hind III Gγ, Hind III Aγ, Hinc II 5'ψβ, Hinc II 3'ψβ, Ava II β, Hinf I 3'-β in the beta globin gene cluster. The PCR amplified products containing each of these polymorphic sites were analysed by digested with the appropriate restriction enzyme and visualised by agarose gel analysis [25,26]. In the present study 10 haplotypes were compared numbered as: 1) Arab-Indian haplotype; 2) Atypical Arab-Indian; 3) Atypical Benin haplotype; 4) Atypical Bantu haplotype; 5) Atypical Cameroon haplotype; 6) Benin haplotype; 7) Atypical Cameroon/Benin haplotype; 8) Cameroon haplotype; 9) Rare 1 and 10) Rare 2.

STATISTICAL ANALYSIS

Statistical analysis was analysed by using the Statistical Package for the Social Sciences (SPSS) software version 16.0. Firstly, data was analysed for normal distribution and homogeneity of variances assumption according to Shapiro-Wilk test and Levene's test, respectively, when the data showed the normal distribution and met the assumptions for parametric test. Further variable was compared by using the student t-test for two groups. Those groups that did not met the parametric assumptions were further compared by non parametric test by using Mann-Whitney U test and Kruskal-Wallis test. Results were presented as mean±Standard Deviation (SD). A p-value of ≤0.05 was considered statistically significant.

RESULTS

In present study, 51 (51%) males and 49 (49%) females were in sickle cell disease group (SS), while 25 (50%) males and 25 (50%) females were in sickle cell trait group (AS). The mean age of the sickle cell disease patients was 23.84±8.38 years and for sickle cell trait was 26.3±7.37 years.

The maximum HbF level of haplotypes was found in Arab-Indian haplotype (22.71±5.27), followed by atypical Arab-Indian (20.85±3.16), rare 2 (14.70±0.14) haplotype, rare 1 (13.70±1.81) and atypical Cameroon (12.84±0.75) [Table/Fig-1].

A haematological comparison between different haplotypes showed insignificant difference for Hb, HCT, MCV and MCH parameters among SS patients. There was a significant difference (p-value <0.0001) only for HbF concentration between all haplotypes in SS patients [Table/Fig-2].

Haplotypes	HbF (Mean±SD)	Hb (Mean±SD)	HCT (Mean±SD)	MCV (Mean±SD)	MCH (Mean±SD)
Arab-Indian haplotype (n=65)	22.71±5.27*	9.35±2.53	30.57±10.54	91.44±11.26	29.20±6.49
Atypical Arab-Indian (n=11)	20.85±3.16*	9.86±1.80	29.78±9.27	89.27±13.07	30.39±12.68
Atypical Benin (n=3)	9.73±0.72*	7.0±3.14	22.63±10.67	92.43±12.51	30.90±3.32
Atypical Bantu (n=3)	3.83±0.56*	9.20±1.22	31.86±8.76	96.43±7.46	27.73±3.76
Atypical Cameroon (n=5)	12.84±0.75*	8.56±1.91	29.26±8.33	85.08±15.65	27.22±6.01
Benin haplotype (n=3)	7.0±0.26*	7.86±5.83	29.10±24.92	90.86±3.81	28.0±5.54
Atypical Cameroon/Benin (n=4)	9.17±0.45*	11.62±2.71	29.57±6.85	92.65±7.27	26.57±4.69
Cameroon haplotype (n=1)	10.1*	5.6	20.6	78.0	23.5
Rare 1 (n=3)	13.70±1.81*	10.13±1.50	24.63±11.62	95.30±7.52	39.86±23.67
Rare 2 (n=2)	14.70±0.14*	9.0±1.41	31.40±8.06	95.55±6.15	29.85±1.62
p-value	<0.001*	0.324	0.822	0.344	0.510

[Table/Fig-1]: The β-globin gene haplotypes with their haematological parameter among sickle cell patients.
*p-value ≤0.05 was considered statistically significant; t=significant (2-tailed) Comparisons were made by independent sample t-test with levene's test for equality of variance
HbF: Fetal haemoglobin (%); Hb: Haemoglobin (g/dL); HCT: Haematocrit (%); MCV: Mean corpuscular volume (fl-femtoliter); MCH: Mean corpuscular haemoglobin (pg-picograms per cell)

Significance between haplotypes	HbF (p-value)	Hb (p-value)	HCT (p-value)	MCV (p-value)	MCH (p-value)
1-2	0.124	0.429	0.801	0.612	0.767
1-3	<0.001*	0.323	0.326	0.905	0.479
1-4	<0.001*	0.855	0.825	0.367	0.578
1-5	<0.001*	0.422	0.753	0.419	0.514
1-6	<0.001*	0.702	0.928	0.835	0.749
1-7	<0.001*	0.193	0.799	0.773	0.353
1-8	<0.021*	0.147	0.352	0.240	0.387
1-9	<0.002*	0.471	0.471	0.473	0.517
1-10	<0.001*	0.784	0.909	0.511	0.686
2-3	<0.001*	0.251	0.371	0.724	0.907
2-4	<0.001*	0.493	0.740	0.267	0.558
2-5	<0.001*	0.238	0.913	0.619	0.509
2-6	<0.001*	0.625	0.967	0.731	0.645
2-7	<0.001*	0.294	0.964	0.543	0.411
2-8	<0.009*	<0.047*	0.365	0.428	0.614
2-9	<0.003*	0.806	0.534	0.345	0.565
2-10	<0.001*	0.541	0.829	0.361	0.895

[Table/Fig-2]: Association of haematological parameter between haplotypes among sickle cell patients.
*p-value ≤0.05 was considered statistically significant; Comparisons were made by independent sample t' test with levene's test for equality of variance
HbF: Foetal haemoglobin (%); Hb: Haemoglobin (g/dL); HCT: Haematocrit (%); MCV: Mean corpuscular volume (fl); MCH: Mean corpuscular haemoglobin (pg)

Mean HbF levels in sickle cell trait were 1.96±0.43 for Arab-Indian haplotype, 0.94±0.19 for atypical Arab-Indian, 0.14±0.05 for atypical Benin, 0.42±0.13 for atypical Cameroon, 0.11±0.01 for rare 1 and 0.2 for rare 2 haplotype [Table/Fig-3].

No significant differences in the mean levels of Hb, HCT, MCV and MCH were observed between haplotypes among sickle cell trait (AS). However, the significant difference (p-value <0.001) was observed only for HbF level between haplotype in AS individuals [Table/Fig-4].

A significant difference was observed for HbF level, Hb and MCV values for Arab-Indian and Atypical Arab-Indian haplotype, while in Atypical Cameroon and Atypical Benin haplotype show significant difference only for Hb and HbF level between AS and SS patient. The result with Arab-Indian haplotype was found significant difference for HbF (p-value <0.001), Hb (p-value <0.002) and MCV (p-value <0.001), Atypical Arab-Indian haplotype for HbF (p-value <0.001), Hb (p-value <0.006) and MCV (p-value <0.023), Atypical Benin haplotype for HbF (p-value <0.017), Atypical Cameroon haplotype for HbF level (p-value <0.008) and Hb (p-value <0.032), respectively. Additionally, no significant difference was observed for rare 1 and rare 2 atypical haplotype between the SS and AS groups [Table/Fig-5].

DISCUSSION

In this study, the haematological differences between β-globin gene haplotypes among sickle cell disease and sickle cell trait patients were examined. The Arab-Indian haplotype was the most common haplotype encountered, followed by atypical Arab-Indian. Due to small number of other atypical haplotype found in this study the

Haplotypes	HbF (%)	Hb (g/dL)	HCT (%)	MCV (fl)	MCH (pg)
Arab-Indian haplotype (n=16)	1.96±0.43*	11.38±1.81	30.70±5.21	74.31±12.66	26.38±4.23
Atypical Arab-Indian (n=19)	0.94±0.19*	12.56±3.33	33.68±5.36	78.65±9.91	27.83±3.22
Atypical Benin (n=7)	0.14±0.05*	11.21±2.07	32.05±4.68	80.25±9.80	33.31±12.80
Atypical Cameroon (n=5)	0.42±0.13*	12.62±2.78	33.76±8.41	83.28±6.11	28.92±2.33
Rare 1 (n=2)	0.11±0.01*	13.20±1.27	26.50±3.46	83.55±7.56	27.75±3.18
Rare 2 (n=1)	0.2*	12.1	27.0	65.5	32
p-value	<0.001*	0.529	0.376	0.288	0.212

[Table/Fig-3]: The β-globin gene haplotypes with their haematological parameter among sickle cell trait (AS) in present study.
*p-value ≤0.05% was considered statistically significant. t=significant (2-tailed). Comparisons were made by independent sample t-test with levene's test for equality of variance
HbF: Foetal haemoglobin (%); Hb: Haemoglobin (g/dL); HCT: Haematocrit (%); MCV: Mean corpuscular volume (fl); MCH: Mean corpuscular haemoglobin (pg)

Significance between haplotypes	HbF (p-value)	Hb (p-value)	HCT (p-value)	MCV (p-value)	MCH (p-value)
1-2	<0.001*	0.194	0.106	0.276	0.271
1-3	<0.001*	0.857	0.550	0.242	0.207
1-4	<0.001*	0.392	0.478	0.049	0.112
1-5	<0.001*	0.246	0.276	0.288	0.655
1-6	<0.001*	0.766	0.501	0.510	0.218
2-3	<0.001*	0.234	0.465	0.714	0.304
2-4	<0.001*	0.970	0.985	0.221	0.420
2-5	<0.001*	0.629	0.147	0.520	0.976
2-6	<0.002*	0.894	0.240	0.213	0.224

[Table/Fig-4]: Association of haematological parameter between haplotypes among sickle cell trait (AS) in present study.
*p-value ≤0.05 was considered statistically significant; Comparisons were made by independent sample t-test with levene's test for equality of variance
HbF: Foetal haemoglobin; Hb: Haemoglobin; HCT: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin

Haplotypes	Patients	HbF	Hb	HCT	MCV	MCH
Arab-Indian	SS (n=65) AS (n=16)	22.71±5.27 1.96±0.43	9.35±2.53 11.38±1.81	30.57±10.54 30.70±5.21	91.44±11.26 74.31±12.66	29.20±6.49 26.38±4.23
	p-value	<0.001*	<0.002*	0.618	<0.001*	0.115
Atypical Arab-Indian	SS (n=11) AS (n=19)	20.85±3.16 0.94±0.19	9.86±1.80 12.56±3.33	29.78±9.27 33.68±5.36	89.27±13.07 78.65±9.91	30.39±12.68 27.83±3.22
	p-value	<0.001*	<0.006*	0.094	<0.023*	0.735
Atypical Benin	SS (n=3) AS (n=7)	9.73±0.72 0.14±0.05	7.0±3.14 11.21±2.07	22.63±10.67 32.05±4.68	92.43±12.51 80.25±9.80	30.90±3.32 33.31±12.80
	p-value	<0.017*	0.117	0.383	0.183	0.833
Atypical Cameroon	SS (n=5) AS (n=5)	12.84±0.75* 0.42±0.13*	8.56±1.91 12.62±2.78	29.26±8.33 33.76±8.41	85.08±15.65 83.28±6.11	27.22±6.01 28.92±2.33
	p-value	<0.008*	<0.032*	0.548	0.690	1.000
Rare 1	SS (n=3) AS (n=2)	13.70±1.81* 0.11±0.01*	10.13±1.50 13.20±1.27	24.63±11.62 26.50±3.46	95.30±7.52 83.55±7.56	39.86±23.67 27.75±3.18
	p-value	0.200	0.200	1.00	0.400	0.800
Rare 2	SS (n=2) AS (n=1)	14.70±0.14 0.2	9.0±1.41 12.1	31.40±8.06 27.0	95.55±6.15 65.5	29.85±1.62 32
	p-value	0.667	0.667	1.00	0.667	0.667

[Table/Fig-5]: Comparison of haplotypes with haematological parameter between AS and SS patients in present study.
*p-value ≤0.05 was considered statistically significant. Comparisons were made by Mann-Whitney U test
HbF: Foetal haemoglobin; Hb: Haemoglobin; HCT: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin

haematological parameters were compared between most frequent found haplotypes and other haplotypes. This is in accordance with the recent studies on Benin haplotype was the most predominant in Nigerian sickle cell patients [13]. In the current study, no significant differences were observed between haematological parameter and β-globin gene haplotypes except for HbF levels.

Foetal haemoglobin is related to the haplotype and correlate with clinical course of Sickle Cell Anaemia (SCA) [27]. Senegal and Arab-Indian haplotypes, by producing the highest levels of HbF in the blood, are associated with less severe clinical evolution of sickle cell anaemia, with a lower occurrence of organic damage [12,28]. As to the Benin and Cameroon haplotypes, the clinical picture is off intermediate severity. The Bantu or Central African Republic (CAR) haplotype is associated with greater clinical severity [29].

Several studies confirmed the association of β-globin gene haplotypes as modulators for clinical presentation of sickle cell disease [27,28]. The Arab-Indian haplotypes influence the disease severity in SCA patients [30-33]. The Benin haplotype is generally associated with lower HbF levels and a severe disease presentation. Elevated HbF levels clearly play a role in decreasing clinical severity, possibly through interfering with HbS sickling process [2,34-36].

Despite the fact some haematological parameters are associated with disease severity of SCD patients [5,24]. In the present study, Hb, HCT, MCV and MCH values were not significantly distributed between haplotypes both in AS and SS patients, probably due to the fact that the studied patients presented a variable clinical course like painful episode, vaso-occlusive crises, jaundice, splenomegaly, anaemia and infection. This is in accordance with the previous studies [27,29,34].

The present findings are in agreement with others studies, which reported similar results for the association of haematological data among the most frequent haplotypes, only MCV had statistical significance in CAR/Benin haplotypes [13,22]. The sickle cell patients showing the severe normocytic normochromic anaemia were associated with reticulocytosis and leucocytosis, thus the haematological parameters like MCV, MCH and HCT were higher in SS patients [37]. Recent study also shows that no significant association was established between the haematological parameters and the various haplotypes; it was observed that patients with the BEN/SEN (Senegal) haplotype displayed a three-fold increased association with improved Hb levels when compared with the BEN/BEN haplotype [13].

In addition, highest HbF level were found in Arab-Indian haplotypes followed by in Atypical Arab-Indian haplotype. These observations suggested that clinical course affect the haematological parameter beside the fact that HbF level is still elevated in SS patients in this study. Beta globin gene haplotypes has been used as a marker for phenotypic heterogeneity of sickle cell disease because of its association with variable HbF levels. However, influence of the Haemoglobin Beta Globin (HBB) gene locus and the XmnI-HBG2 site on HbF levels in SCA has been validated by many studies in several populations [33,35,36].

In SCA patients with Bantu haplotype often have low HbF levels, while those with the Cameroon and Benin haplotypes show intermediary HbF levels. However, the present results agree with some previous published data on HbF levels and β^S-globin haplotype [18]. In this study, the presence of intermediate HbF levels for the Cameroon haplotype could be due to sequence variations in regulatory regions, such as the 5'HS2 and flanking region of the γ gene [38,39].

Limitation(s)

The limitations of this study were the other genetic polymorphisms that might associate with disease severity were not studied, which can be included in the further studies to determine the correlation with disease severity.

CONCLUSION(S)

In the present study, HbF level was significantly associated with haematological parameters and β-globin haplotypes. High HbF level and β-globin gene cluster haplotypes act as modulators for disease severity of sickle cell patients of Chhattisgarh, India. More studies as modulators for sickle cell disease are essential to find the disease severity that can be helpful for treatment and management of sickle cell disease.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jun 24, 2022
- Manual Googling: Oct 31, 2022
- iThenticate Software: Nov 07, 2022 (18%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

Date of Submission: **Jun 08, 2022**
Date of Peer Review: **Jul 22, 2022**
Date of Acceptance: **Nov 08, 2022**
Date of Publishing: **Feb 01, 2023**