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Coinheritance of -3.7 Alpha-Thalassaemia Deletions Among Sickle Cell Anaemia Patients in Chhattisgarh, India

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Abstract

Background: Alpha (α)-thalassaemia is inherited as an autosomal recessive disorder characterised by microcytic hypochromic anaemia. In children with homozygous sickle cell anaemia (SCA), coinheritance of α-thalassemia reduces the risk of cerebral vasculopathy and alters the disease severity. This study aimed to detect the occurrence of α-thalassemia in homozygous SCA patients in Chhattisgarh state (India) and to evaluate their clinical and haematological profiles. ***Methods:*** In this study, a total of 203 well-characterized homozygous SCA patients who were diagnosed by Hb electrophoresis and Restriction Fragment Length Polymorphism (RFLP) methods were included. Genotypes of most common two deletional mutations of the α-thalassaemia genes were screened using multiplex gap-PCR. The calculation of gene frequency for α-thalassaemia mutations was done based on the gene counting method. ***Results:*** The average age at enrolment was 12.1 years for SCA. The average fetal haemoglobin was 18.7%. Out of 203 SCA patients, 9.85% and 5.4% were heterozygous ($-\alpha^{3.7}/\alpha\alpha$) and homozygous ($-\alpha^{3.7}/-\alpha^{3.7}$) for the α-thalassemia 3.7 kb deletions respectively and this distribution deviated from Hardy-Weinberg equation. There were significant differences in MCH values found in SCA with and without α-thalassaemia. Symptoms like abdominal pain and headache were significantly different between SCA with and without α-thalassaemia. **Conclusions**: In SCA patients coinheriting α-thalassemia, the clinical and laboratory results indicated improvement in overall clinical presentation of disease. Coinheritance of -3.7 alpha-thalassaemia deletions among sickle cell anaemia patients significantly resulted in milder clinical course.

Keywords: Sickle cell anaemia, Alpha thalassaemia, Co-inheritance, Genetic analysis, α-3.7 deletion

Introduction

Sickle cell anaemia (SCA) is a severe monogenic genetic disorder occurs because of a mutation in the haemoglobin gene and is associated with erythrocytes sickling. SCA is more common in Africa, America, the Mediterranean region, Middle Eastern countries and the Indian subcontinent (Bhaskar and Patra, 2015). In Equatorial Africa, the prevalence of sickle cell anaemia ranges from 10-40%, whereas in West African countries, the range is 15-30% (Uyoga *et al.*, 2019). In 1952 Lehmann and Catbush reported the first case of SCA in the tribal population of the Nilgiri hills of Indian subcontinent (Lehmann and Cutbush, 1952). A study conducted by Indian Council of Medical Research (ICMR) in Maharashtra, Orissa, Andhra Pradesh and Tamil Nadu revealed the prevalence of HbS allele as 5-34% in these populations (Jain *et al.*, 2013; Tewari and Rees, 2013). Sickle cell anaemia is more prevalent in Chhattisgarh populations and some communities; the prevalence of the sickle cell trait is as high as 30%. However, the frequency of the homozygous SCA in Chhattisgarh is 0.21 % (Patra *et al.*, 2015).

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Pain and vaso-occlusive crisis (VOC) are the major clinical manifestations of sickle cell anaemia leading to various complications as well as multi-organ failure and death (Lakkakula *et al.*, 2017; Shukla *et al.*, 2017; Lakkakula *et al.*, 2018; Bhaskar, 2019; Lakkakula, 2019). But it can also depend on the haplotypes, for example the Indo-Arab haplotype is milder due to the higher fetal haemoglobin (HbF) level (Nongbri *et al.*, 2017). The HbF induction is extremely pleiotropic and influenced by many genes, such as BCL11A, KLF-1 and HBS1L-MYB (Bhanushali *et al.*, 2015). Coinheritance of other haemoglobinopathies is known to substantially modulate the clinical manifestations of SCA (Saleh-Gohari and Mohammadi-Anaie, 2012; Jha *et al.*, 2018).

Alpha-thalassaemia is caused by the down-regulation of α-globin chain synthesis, which leads to reduced production of both fetal (HbF) and adult (HbA/HbA2) heamoglobins (Farashi and Harteveld, 2018). Deletions involving one or both α-genes are responsible for the majority of the α-thalassemia cases, while non-deletional mutations account for the minority. Several lines of evidence show that coinheritance of α- thalassemia has an advantageous effect on the survival of patients with SCA. Since hematopoietic stem cell transplantation (HSCT) is only curative but not feasible to all SCA patients, a myelosuppressive agent, like hydroxyurea is the only effective drug that has been shown to reduce the frequency of painful episodes (Verma *et al.*, 2018). It has been reported that the patients with SCA and α-thalassemia had higher haemoglobin (Hb) levels, RBC counts, and HbA2 levels, with lower reticulocyte counts, MCV, MCH, and HbF levels than those with a normal α-genotype (Olatunya *et al.*, 2019). In addition, patients with α-thalassaemia and SCA are associated with improved haematological indices, lower consultations rate and hospital admissions (Rumaney *et al.*, 2014). These clinical diversities suggest a biological interaction between SCA and α-thalassaemia with their resultant haematological effects (Birgens and Ljung, 2007). Hence, this study aimed to evaluate the clinical and haematological profiles of SCA patients coinheriting with α-globin gene deletions in Chhattisgarh state (India) where SCA prevalence is high.

Materials and methods

Study population: The present study was performed under the Institutional Ethics Committee of the Pt. J. N. M. Medical College, Raipur. The study population consisted of SCA patients attending the outpatient department of the Sickle Cell Institute Chhattisgarh, Raipur. Informed written consents were obtained from all study participants. A total of 203 homozygous SCA subjects were diagnosed by Hb electrophoresis and RFLP methods. Briefly, in RFLP, in order to classify the differences in DNA sequences, a DdeI restriction enzyme had been used to digest the PCR product by incubating at 37 °C. Based on the size variations of the digested fragments, the differentiation of the homozygous, heterozygous SCA and normal subjects were made. Blood samples collected before initiating hydroxyurea treatment were used to analyse complete blood count (CBC), HPLC and DNA analysis. In this study, all selected homozygous SCA patients were more than 5 years of age.

Haematology parameters: About 3ml blood samples were collected in EDTA coated vacutainers for CBC and Hb analysis. Measurement of CBC was done by a three-part haematology analyser (Cellenium19; Trivitron Health Care, Chennai, Tamil Nady, India), while the HPLC analysis was done by HB variant machine (Bio-Rad Laboratories, Hercules, CA, USA). Clinical features were assessed and documented for each study subject. Information on demographics, medical history, and other factors were obtained from each participants' medical records. Individuals who had been transfused in the previous 3 months were excluded from the study.

Identification of alpha gene deletions: Genomic DNA was extracted using the modified salting-out method. The multiplexed GAP-PCR assay was used to detect –3.7 and -4.2 deletional alpha thalassaemia. The following 5 primers (1) α-2/3.7-F: CCCCTCGCCAAGTCCACCC, (2) 3.7/20.5-R: AAAGCACTCTAGGGTCCAGC G, (3) α-2-R: AGACCAGGAAGGGCCGGTG, (4) 4.2-R: CCCGTTGGATCTTCTC ATTTCCC, (5) 4.2-F: GGTTTACCCATGTGGTCTC that optimized for detecting α-thalassemia deletions were taken from a previous study (Old *et al.*, 2012). Briefly, multiplex GAP-PCR was performed in a total reaction volume of 20 µl, containing 2×PCR buffer, 10 µM of each primer, 25 mM Magnesium Chloride, 0.3 mM dNTPs (Fermentas), 0.5 units of Taq DNA polymerase (Merck), 5M Betaine, 10% DMSO and 50 ng of the genomic DNA. The reaction mixture was amplified for 30 cycles with denaturation at 95 °C for one minute, annealing at 64 °C for one minute and extension at 72 °C for two minutes & 10 minutes using a thermal cycler (Applied Biosystems, USA). To score the genotypes, amplified PCR products were electrophoresed through 1.5 % agarose gel and visualized using gel documentation instrument (Omega 16vS Molecular Imaging and Analysis

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System) (Figure 1). The calculation of gene frequency for α-thalassaemia mutations was done based on the gene counting method. Hardy-Weinberg proportions for α-thalassaemia deletions were also calculated. Differences in continuous variables were determined by parametric (Student's t-test). Interaction analysis between haemoglobin, clinical signs and symptoms in SCA patients with or without α-thalassaemia was performed using MH analysis. All statistical analysis were performed using SPSS software version 20.0 (SPSS Inc, Chicago, Illinois) for Windows.

Results

An analysis of 203 SCA patients using multiplex GAP-PCR revealed presence of -$\alpha^{3.7}$/αα (heterozygous deletion) in 20 (9.85%) individualsand -$\alpha^{3.7}$/-$\alpha^{3.7}$ (homozygous deletion) in 11 (5.42%) individuals (Figure 2).

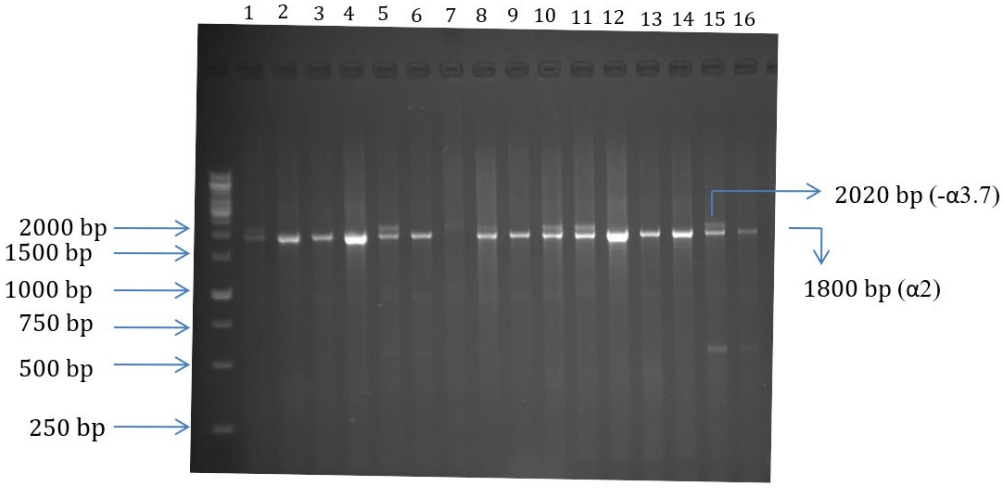

Figure 1. Gel picture of alpha gene deletions of the present study (Lane 1, 5,8, 10,11 and 15 are showing heterozygous -$\alpha^{3.7}$/αα deletion and Lane 2, 3, 4, 6, 9, 12, 13, 14 and 16 are homozygouse -$\alpha^{3.7}$/-$\alpha^{3.7}$ deletion).

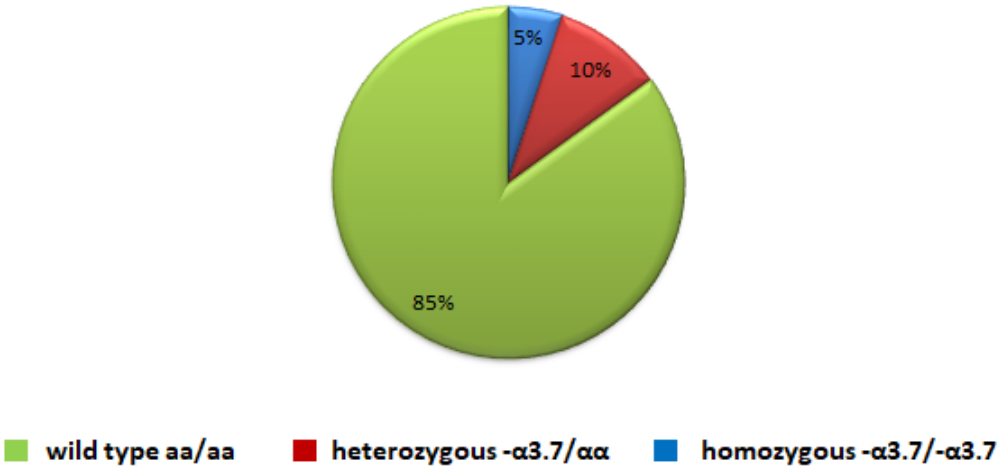

Figure 2. Distribution of alpha thalassemia deletion in the study population.

There were no $-\alpha^{4.2}$ deletions found among the study subjects. In addition, α-gene deletion genotype results of this study did not follow the Hardy-Weinberg equilibrium. ($p<0.001$).

The values pertaining to the analysis of CBC and other biochemical variables in SCA with and without α-thalassemia were documented in Table 1. A comparison of CBC values between these two groups revealed that there were no significant differences in RBC, WBC, platelets, and haematocrit levels. The HbF and HbS levels also did not show any significant differences between both groups. However, MCV and MCH values were significantly different between these groups.

Table 1. Distribution of hematological and biochemical variables among SCD patients with or without alpha thalassemia.

Variable	**SCA with α thalassemia mutations ($-\alpha^{3.7}/\alpha\alpha$ and $-\alpha^{3.7}/-\alpha^{3.7}$)**	**SCA without α thalassemia mutation (αα/αα)**	**P value**
Hb (g/dl)	8.77±2.29	8.27±1.71	0.152
HbF	18.31±3.12	20.2±4.13	0.135
HbS	74.28± 5.4	72.20± 7.2	0.127
WBC ($x10^3/\mu l$)	10.03±2.81	10.02±3.77	0.99
Haematocrit (%)	27.23±7.14	25.38±5.62	0.109
Platelet count ($x10^3/\mu l$)	283.10±117.85	312.88±149.36	0.294
RBC ($x10^6/\mu l$)	3.42±1.15	3.15±1.77	0.408
MCV (fl)	80.13±8.71	85.73±13.58	0.028
MCH (pg)	25.61±4.10	27.93±4.82	0.013
MCHC (g/dl)	32.03±3.60	32.49±3.39	0.495
RDW-CV (%)	19.03±3.98	18.24±2.90	0.188
TB (mg/dl)	1.48±1.26	1.91±1.22	0.075
DB (mg/dl)	0.26±0.44	0.41±0.49	0.105
SGOT (IU/L)	29.58±15.51	33.83±17.31	0.203
SGPT (IU/L)	19.74±11.98	25.19±15.45	0.064
Blood urea (mg/dl)	15.84±1.86	16.98±3.28	0.061
Serum creatinine (mg/dl)	0.52±0.51	0.56±0.50	0.667
Serum sodium (meq/L)	133.65±1.89	132.66±10.07	0.588
Serum potassium (meq/L)	3.29±0.46	3.30±0.46	0.945

Hb: hemoglobin; **WBC**: white blood cells; **RBC**: red blood cells; **MCV**: mean corpuscular volume; **MCH**: mean corpuscular hemoglobin; **MCHC**: mean corpuscular haemoglobin concentration; **RDW**: red cell distribution width; **TB**: total bilirubin; **DB**: direct bilirubin; **SGOT**: serum glutamic oxaloacetic transaminase; **SGPT**: serum glutamic-pyruvic transaminase

Values related to liver function tests such as total bilirubin, direct bilirubin, SGOT and SGPTs did not show significant differences between these groups. Other variables which were blood urea, Clinical signs and symptoms related to sickle cell anaemia found in the study subjects were described in Table 2. Clinical symptoms of abdominal pain and headache showed significant difference between SCA with and without α-thalassemia groups. Coinheritance of α-thalassemia abnormalities in SCA patients drastically reduced the rate of blood transfusion requirement and the need for hospitalization. serum creatinine, serum sodium and serum potassium were not significantly different between these two groups.

Clinical severity in terms of abdominal pain and headache were significantly diverse in different anaemia groups and exhibited a confounding effect on the relationship between clinical severity and α-thalassemia (Table 3). However, clinical signs and symptoms such as leg ulcers, oedema, haemolytic face and priapism were not seen in our study subjects.

Table 2. Clinical phenotype between SCD patients with or without alpha thalassemia.

	SCA with α thalassemia mutations ($-\alpha^{3.7}/\alpha\alpha$ and $-\alpha^{3.7}/-\alpha^{3.7}$)		**SCA without α thalassemia mutation ($\alpha\alpha/\alpha\alpha$)**		**Total**	**P-Value**
Phenotype	**No**	**Yes**	**No**	**Yes**		
Pallor	18	13	69	101	201	0.161
Icterus	23	8	107	65	203	0.201
Fever	18	13	98	73	202	0.911
Body Ache	20	11	104	67	202	0.847
Pain Abdomen	30	1	124	47	202	0.013
Headache	29	2	130	42	203	0.025
Bony tenderness	30	1	164	8	203	0.723
Hepatomegaly	31	0	163	9	203	0.193
Splenomegaly	26	5	146	26	203	0.888
Dactylitis	31	0	169	3	202	0.459
Pain In Hip	30	1	164	8	203	0.723
Difficulty In Walking	30	1	164	8	203	0.723
Blood Transfusion	30	1	143	23	197	0.107
Hospitalization	30	1	146	26	203	0.073
Oedema	31	0	172	0	203	-
Leg Ulcer	31	0	172	0	203	-
Haemolytic Face	31	0	172	0	203	-
Priapism	31	0	172	0	203	-

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Table 3. Association between different symptoms and hemoglobin stratified by Sickle cell disease patients with or without alpha thalassemia.

Hb status	SCD status	Clinical symptom		OR (95% CI)	P value
		No	Yes		
Abdomen Pain					
Hb <7	**SCD with α thalassemia**	8	0	-	<0.001
	SCD without α thalassemia	15	38		
Hb >7	**SCD with α thalassemia**	22	1	1.8 (0.22-14.94)	1.00
	SCD without α thalassemia	110	9		
M-H interaction				8.23 (1.42-47.83)	0.003
Headache					
Hb <7	**SCD with α thalassemia**	6	2	8.35 (1.51-46.33)	0.012
	SCD without α thalassemia	14	39		
Hb >7	**SCD with α thalassemia**	23	0	-	1.0
	SCD without α thalassemia	116	3		
M-H interaction				9.42 (1.63-54.52)	0.016

Hb: hemoglobin**; OR:** odds ratio**; CI:** confidence interval**; SCD:** sickle cell disease

Discussion

Among the 203 SCA study subjects, 15.27% of them showed presence of 3.7 α-deletion. The distribution of α-gene deletions fail to follow the Hardy Weinberg equation. A comparison of CBC in SCA with and without α-thalassemia groups showed significant differences in MCH values. There were no clinical symptoms and signs such as leg ulcers, oedema, haemolytic face, and priapism found in our study subjects. Interaction analysis showed that the pain in the abdomen and headache were significantly increased in SCA subjects with α-thalassemia deletions as compared to those without α-thalassemia deletions and Hb<7 gm/dl.

Coinheritance of α-thalassemia is one of the major determinants of clinical severity in SCA patients (Tewari and Rees, 2013). The $-\alpha^{3.7}$ deletion is the most prevalent in sub-Saharan Africans, among whom point mutations have been seldom reported in the α-globin gene (Purohit *et al.*, 2014). In the present study, only 3 $-\alpha^{3.7}$ deletions were found with the gene frequency of (-α-) 5.17%, which was higher than the frequency that had been reported from many places in India and abroad. The high coinheritance of sickle cell trait and α-globin gene deletions was expected since α-globin deletion was previously reported as the most common globin gene mutation in previous studies around the world (Saleh-Gohari and Khosravi-Mashizi, 2010; Borgio *et al.*, 2018; Cartín-Sánchez *et al.*, 2019). In contrast to a study from North India, our study results showed there were no $-\alpha^{4.2}$ deletions found among the study subjects (Sharma *et al.*, 2015). An analysis of 75 patients from the Shahdol district of Madhya Pradesh region showed 43% of their study subjects had $-\alpha^{3.7}$ deletions (Dubey *et al.*, 2013). Meanwhile in Odisha population, both $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions had been identified (Purohit, Dehury et al., 2014). Ten percent of 133 non-tribal Bengali patients showed the presence of $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions. (Rudra *et al.*, 2008). Lastly, a study from Madhya Pradesh showed 41.3% of SCA patients had α-thalassemia (Singh *et al.*, 2016).

Several studies had described the variation in the haematological profile of SCA individuals with α-thalassaemias. A moderate reduction in MCV and

Hb were found in heterozygous (-$\alpha^{3.7}$/αα) and homozygous (-$\alpha^{3.7}$/-$\alpha^{3.7}$) α- thalassemia patients (Williams *et al.*, 1996). As higher level of haemoglobin makes patients more prone to vaso-occlusive and painful crisis, reduced Hb in SCA patients inheriting α-thalassemia causes a reduction in haemolysis (Rahim *et al.*, 2013; Lubega *et al.*, 2015). The coexistence of α-thalassemia with SCA significantly improves haematological parameters and results in a lower need for blood transfusions (Pandey *et al.*, 2011). The coexistence of α-thalassemia with SCA also showed reduced musculoskeletal pain and cerebrovascular disease risk in children with SCA (Belisario *et al.*, 2010; Vaz *et al.*, 2011). The variations observed in the cellular pathology are probably due to an imbalance between α- and β-globin chain production and an excess of β-globin chains (Wambua *et al.*, 2006; Srinoun *et al.*, 2009).

Although α-gene deletion identification in SCA patients is the primary objective of this study, the present study concentrated only on –$\alpha^{3.7}$ and –$\alpha^{4.2}$ deletions and does not consider SEA, FIL, and MED α-gene deletions. This is one of the main limitations of this study. However, to the best of our knowledge, this is the first study that showed the coinheritance of SCA with α- thalassemia in the Chhattisgarh population. As the haemoglobinopathies impose a significant burden on health resources as well as psychological effects on families, educational and premarital screening programs should be offered to the high-risk population groups.

Abbreviations

Sickle cell anaemia: SCA
Indian Council of Medical Research: ICMR
Fetal Hemoglobin: HbF
Complete blood count: CBC

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Competing interests

The authors declare that they have no competing interests.

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