

Original Research Article

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Prevalence of Thalassaemia Mutations in Sickle Cell Disease Population of Madhya Pradesh, Central India

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ABSTRACT

About one third of sickle cell anemia patients have coexisting alpha thalassaemia and it is an important genetic determinant of hematologic severity in sickle-cell disease. Alpha-thalassaemia reduces polymerization of sickle hemoglobin in homozygous sickle cell disease. Present study aims to identify the prevalence of alpha and beta-thalassaemia mutations in the sickle cell disease subjects and its effect on red cell indices. A total of 300 samples of homozygous sickle cell anemia (SCA) and 60 of sickle-beta-thalassaemia (SBT) subjects in Madhya Pradesh were screened for thalassaemia mutations. The $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions of alpha thalassaemia and five common beta-thalassaemia mutations were characterized by PCR. The overall prevalence of alpha-thalassaemia was 41.3% in SCA and 35.0% in SBT while 40.2% in general (SCA+SBT). Heterozygous alpha-thalassaemia ($-\alpha/\alpha\alpha$) and $-\alpha^{3.7}$ deletions were predominant in the two categories (SCA and SBT). The tribal (ST) populations were found to have higher prevalence of alpha-thalassaemia (61.9% in SCA & 100% in SBT). Among beta-thalassaemia mutations, IVS1-5 (G→C) was most common (80.0%) followed by Codon15 (G→A), Codon 8/9 (+G) and Codon41/42 (-TCTT). More than one third of sickle cell disease individuals tested in the study carried the α -deletions which can be used as the predictor of disease severity.

Keywords

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Introduction

Sickle hemoglobin (HbS) is caused by a single nucleotide substitution (A → T) in the 6th codon of β -globin gene on chromosome 11 which results in replacement of Glutamic acid (GAG) by Valine (GTG) (Pauling *et al.*, 1949; Ingram 1956). Under deoxygenated conditions, this substitution causes HbS polymerization (Bunn, 1997)

and modifies the stability of the hemoglobin leading to the clinical disorder. The homozygous state of the sickle gene (β^S) results in sickle cell anemia (SCA) and is responsible for the most common severe variant of Sickle cell disease. Thalassaemias occur due to reduced or no hemoglobin production from lack of synthesis of alpha or beta chains. Alpha thalassaemia (α -

thalassaemia) is classified into the α^0 -thalassaemia when no α -chain is produced and α^+ -thalassaemia when synthesis of α -chain is reduced (Weatherall *et al.*, 2001a). The α^+ -thalassaemia is commonly caused by the deletion of one of the linked pair of α -globin genes. The common deletions which cause α^+ -thalassaemia are $-\alpha^{3.7}$ deletion (rightward deletion) and $-\alpha^{4.2}$ deletion (leftward deletion) that remove one or more of the duplicated structural α -genes (Embury *et al.*, 1980; Higgs, 2013). The frequency of α^+ -thalassaemia varies from 10–20% in sub-Saharan Africa, over 40% in Middle Eastern countries and up to 80% in India and northern Papua New Guinea (Weatherall *et al.*, 2001b; Singh *et al.*, 2016) and these frequencies show a strong correlation with malarial endemicity (Flint *et al.*, 1986).

Beta thalassaemia (β -thalassaemia) is also classified into the β^+ -thalassaemia wherein synthesis of β -chains are reduced and β^0 -thalassaemia when no synthesis of β -chains occurs (Weatherall, 2001). The prevalence of β -thalassaemia varies from 1% to 20% in Mediterranean basin and some parts of Africa, the Middle East, South-East Asia, India, Melanesia and the Pacific Islands (Weatherall *et al.*, 2001b). More than 200 mutations are known to be responsible for β -thalassaemia (Higgs *et al.*, 2012). Among them IVS1-5(G→C), IVS1-1(G→T), 619-bp deletion, Codon 41/42(-TCCT) and Codon 8/9(+G) mutations are responsible for more than 80% of β -thalassaemia cases in India (Sinha *et al.*, 2009). The present study was carried out to identify the prevalence of α^+ -thalassaemia mutations ($-\alpha^{3.7}$ & $-\alpha^{4.2}$) and common β -thalassaemia mutations in the sickle cell disease subjects and their effect on red cell indices. Currently no reports exist indicating the prevalence of alpha and beta thalassaemia mutations in sickle cell disease in Central India.

Materials and Methods

Sample Collection

A total of 360 blood samples of sickle cell disease (SCD) individuals were screened for thalassaemia mutations after obtaining written and understood consent. All these subjects were registered with NIRTH SCD Clinic. Homozygous Sickle cell anemia (300) and Sickle β -thalassaemia (60) individuals were included in this study. All these subjects were referred from various OPD's of NSCB Medical College, Jabalpur to National Institute for Research in tribal health for the diagnosis of hemoglobinopathies in and around Jabalpur district.

Diagnosis Criteria

Diagnosis was established and confirmed by Parental investigation. If both parents were found to be heterozygous for sickle hemoglobin, their child was considered as homozygous sickle cell disease (sickle cell anemia-SCA). If one parent was heterozygous for sickle hemoglobin and other parent has normal hemoglobin pattern with elevated HbA₂ levels, their child was considered as double heterozygous for sickle gene and thalassaemia gene (sickle beta thalassaemia-SBT).

Basic Laboratory Investigations

Hematological parameters (Hb%, MCV, MCH and MCHC) were measured on an automated blood cell counter (Cellenium 19, China). Sickle hemoglobin was identified by sickling test with 2% sodium metabisulphite and confirmed by electrophoresis (Figure-1) on cellulose acetate membrane with TEB buffer (Tris-EDTA-Borate) at pH 8.6 (Chanarin, 1989). HbA₂ was quantified by elution from Cellulose acetate membrane (Dacie *et al.*, 1991).

Molecular Screening by PCR

DNA was extracted by the using genomic DNA purification kit (K0512, Thermo Fisher Scientific Inc.) and Polymerase chain reaction was used to detect $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions (Figur-2 & Figure-3) as described earlier (Baysal *et al.*, 1984). Beta thalassaemia mutations were characterized by ARMS PCR (Mohanty *et al.*, 2008).

Results and Discussion

Molecular analysis of 300 homozygous sickle cell anemia (SCA) and 60 sickle beta thalassaemia (SBT) individuals were carried out for $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions. Table-1 shows the deletion profile of SCA and SBT individuals included in the study. The overall prevalence of α -thalassaemia was 40.2%. Group wise prevalence was 41.3% for SCA and 35.0% for SBT subjects and this difference was statistically not significant.

Majority of homozygous sickle cell anemia patients (30.7%) were heterozygous for α -thalassaemia deletion ($-\alpha/\alpha\alpha$) and 9.3% carried homozygous ($-\alpha/-\alpha$) deletions. The $-\alpha^{3.7}$ deletion was higher (31.6%) than $-\alpha^{4.2}$ deletion (11.0%) while 1.3% showed both deletions ($-\alpha^{3.7}/-\alpha^{4.2}$). As in the case of α -thalassaemia, majority of sickle beta thalassaemia (SBT) individuals also showed $-\alpha/\alpha\alpha$ (30.0%) deletion and only 5.0% were homozygous for $-\alpha/-\alpha$ deletion. SBT subjects showed predominantly $-\alpha^{3.7}$ deletion (25.0%) followed by $-\alpha^{4.2}$ deletion (10.0%). Notably, no double heterozygous condition was encountered among the present SBT study subjects also.

Effort was also made to identify whether the co-existing of α -thalassaemia with sickle cell disease (SCA & SBT) showed any relationship with regard to caste as sickle

cell hemoglobin was found to be associated with communities involved in endogamous marriage practices. Table-2 depicts caste wise analysis of α -thalassaemia mutations in sickle cell disease individuals. Among the SCA, higher number of Scheduled tribe individuals (Gond and Pradhan) carried α -thalassaemia mutations (61.9%) followed by 50.0% in other backward class (Patel, Yadav and Barman) and 34.5% in Scheduled castes (Chadar Deharia, Jharia, Katiya, Mahar, Mehra). About 18% Muslims and Rajputs also showed α -thalassaemia mutations (Table-2). In case of SBT individuals, all Scheduled tribe subjects carried α -thalassaemia followed by 34.8% of other backward class, 18.2% others and Scheduled caste (11.8%) respectively.

Mutational analysis on sixty sickle beta thalassaemia (SBT) study individuals were carried out to identify the presence of common β -thalassaemia mutations such as IVS1-5 (G→C), Codon 15 (G→A), Codon 8/9 (+G), Codon 41/42 (-TCTT), IVS1-1(G→A) and 619-bp deletions. The majority (80.0%) of the studied SBT population were found to carry IVS1-5 (G→C) mutation (Figure-4) and other mutations namely Codon15 (G→A), Codon 8/9 (+G) and Codon 41/42 (-TCTT) were in equal frequency (6.7%). The IVS1-1(G→A) and 619-bp deletion were not detected in the studied subjects. The IVS1-5 (G→C) mutation was found in all Scheduled tribe individuals followed by OBC (87.0%), others (72.7%) and SC (64.7%). The Codon15 (G→A) mutation was found only in SCs (23.5%) whereas Codon 8/9 (+G) was found among Muslims & Rajputs (27.3%) and other backward classes (4.3%) only. Codon 41/42 (-TCTT) was encountered only among Scheduled caste (11.8%) and other backward classes (8.7%).

The effect of α -thalassaemia on red cell

indices of SCA and SBT were evaluated. The mean hemoglobin levels of SCA with or without α -thalassaemia were in the range of moderate anemia and no significant differences were observed either in the hemoglobin levels or HCT (Table-3). On the other hand, mean MCV (P=0.0001) and MCH (P=0.0001) of SCA with α -thalassaemia were significantly lower than non α -thalassaemia individuals. Interestingly, significant increase in mean total red cell count (P=0.0001) was observed in α -thalassaemia subjects when compared with non-thalassaemia group. No significant difference in mean HbF levels was observed between any of the groups. On the other hand, the mean MCV (P=0.0216) and MCH (P=0.0213) of individuals with homozygous (- α /- α) were significantly lower than heterozygous (- α / α) individuals. In contrast, presence or absence α -thalassaemia mutation did not affect hematological indices of SBT individuals.

Alpha thalassaemia is a known major modifier in sickle cell disease presentation (Thein 2008). Identification of the presence of α -deletions in sickle cell disease patients

of any given population is a pre-requisite to recognizing its effect on sickle cell disease presentation. Three hundred and sixty sickle cell subjects were evaluated for α -thalassaemia deletions. About 40.2% of cases were found to carry α -thalassaemia deletions predominantly among tribal individuals of both SCA (61.9%) and SBT (100%) cases. Majority of the individuals were heterozygous (- α / α) for - α ^{3.7} deletion or - α ^{4.2} deletion. The - α ^{3.7} deletion was predominant over - α ^{4.2} deletion. In Africa about one third of sickle cell anemia patients have been shown to carry coexisting α -thalassaemia (- α ^{3.7} deletion) in heterozygous and homozygous states (Steinberg, 2009). Further, sickle cell anaemia patients in Brazil, California and Guadeloupe were found to carry α -thalassaemia (Figueiredo *et al.*, 1996; Kéclard *et al.*, 1996; Schroeder *et al.*, 1989). In India, about 30% of sickle cell anemia patients in a hospital based study, all the tribals in Western India and 32% of non-tribal sickle homozygous individuals were found to carry α -thalassaemia (Sanjay *et al.*, 2011; Mukherjee *et al.*, 1997a).

Table.1 Percent prevalence of α -thalassaemia in sickle cell disease patients of Madhya Pradesh (India)

	No. of Patients	$\alpha\alpha/\alpha\alpha$	- α ^{3.7} /- α ^{3.7}	- α ^{3.7} / $\alpha\alpha$	$\alpha\alpha$ /- α ^{4.2}	- α ^{3.7} /- α ^{4.2}	Total α -thal.
Homozygous sickle cell disease (SCA)	300	58.7	9.3	21.0	9.7	1.3	41.3
Sickle β-thalassaemia (SBT)	60	65.0	5.0	20.0	10.0	0	35.0
Total	360	59.8	8.6	20.8	9.7	1.1	40.2

Table.2 Percent prevalence of α -thalassaemia in sickle cell disease patients and their caste distribution

Caste		No. of Patients	α -thal. (%)
Scheduled caste (194)	SCA	177	34.5
	SBT	17	11.8
Scheduled tribe (51)	SCA	42	61.9
	SBT	9	100.0
Other backward class (93)	SCA	70	50.0
	SBT	23	34.8
Others (22)	SCA	11	18.2
	SBT	11	18.2
Total (360)	SCA	300	41.3
	SBT	60	35.0
* Values in parenthesis are total number of patients			

Table.3 Mean hematological indices of Sickle cell disease and effect of α -thalassemia

Genotype	N	Hb (g/dl) (Mean±SD)	HCT (%) (Mean±SD)	TRBC (X 10 ⁶) (Mean±SD)	MCV (fl) (Mean±SD)	MCH (pg) (Mean±SD)	MCHC (g/dl) (Mean±SD)	HbF (%) (Mean±SD)
$\alpha\alpha/\alpha\alpha$ (SCA)	176	7.8±1.8	24.6±5.4	2.9±0.7	87.3±8.9	27.9±4.0	31.9±2.6	15.5±6.3
α -thal (SCA)	124	7.9±1.6	25.1±4.8	3.3±0.8	78.2±9.2	24.6±3.6	31.8±5.1	14.4±5.6
				P=0.0001	P=0.0001	P=0.0001		
$-\alpha/\alpha\alpha$ (SCA)	92	7.9±1.5	25.1±4.6	3.2±0.7	79.3±9.1	25.1±3.6	31.5±2.5	14.9±5.8
$-\alpha/-\alpha$ (SCA)	32	7.9±1.8	25.3±5.3	3.4±0.9	75.0±8.7	23.4±3.4	31.1±1.8	13.2±4.9
					P=0.0216	P=0.0213		
$\alpha\alpha/\alpha\alpha$ (SBT)	39	7.3±1.6	24.4±5.1	3.7±0.9	68.1±8.1	20.5±3.1	30.1±1.7	17.2±6.2
α -thal (SBT)	21	7.4±1.8	24.4±5.5	3.5±0.9	71.5±7.9	21.7±3.0	30.3±1.7	14.1±5.6

Fig.1 Hemoglobin electrophoresis on cellulose acetate medium

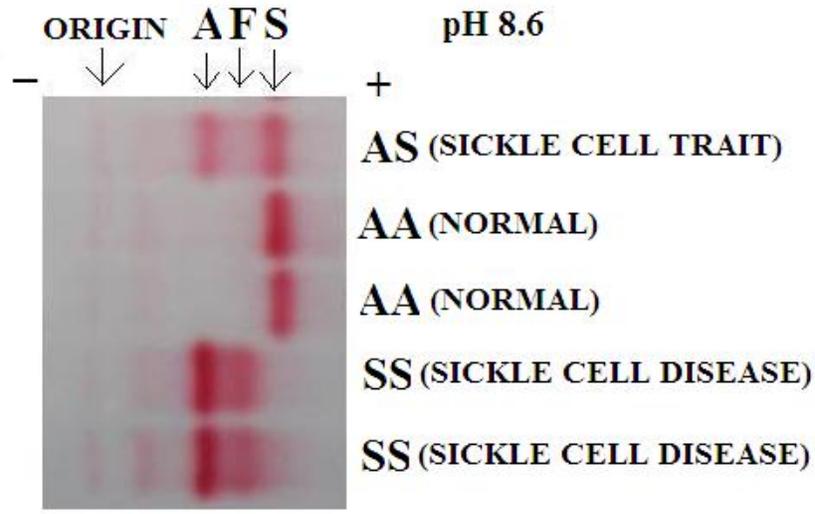


Fig.2 Agarose gel electrophoresis of PCR product to detect -3.7 deletion

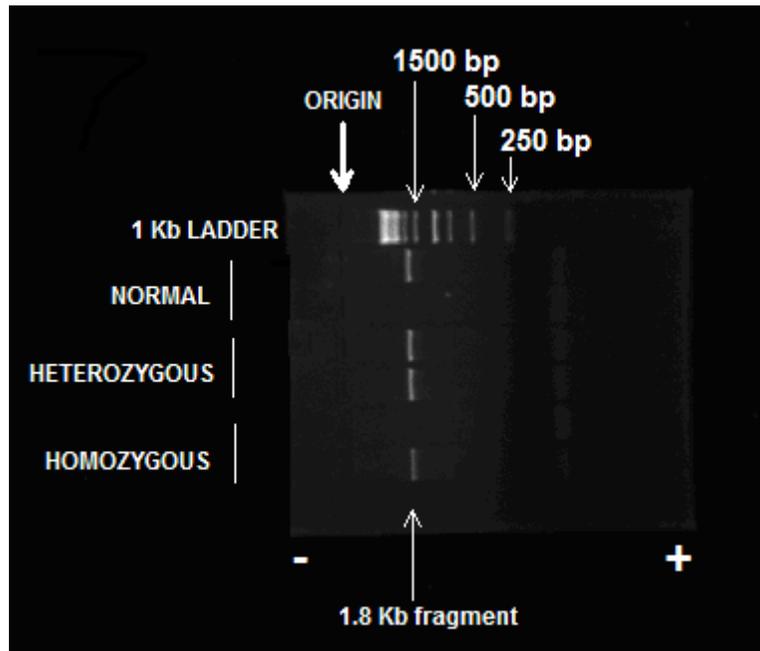
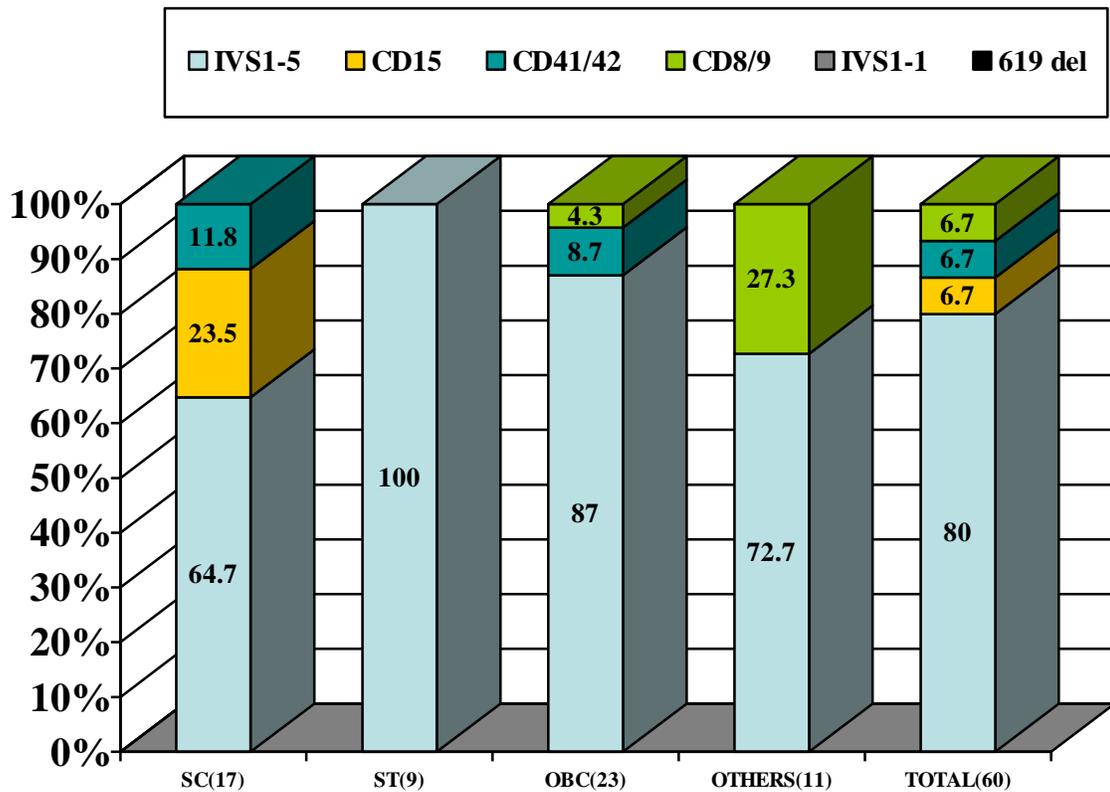


Fig.3 Agarose gel electrophoresis of PCR product to detect -4.2 deletion



Fig.4 Percent prevalence of beta thalassaemia mutations in sickle cell disease patients



Recently, Yadav *et al.*, 2016 reported variation in the clinical phenotype of Sickle cell disease patients with different genotypes. Moreover, it is reported that SCD individuals with α^+ -thalassaemia show higher hemoglobin level but lower mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) with resultant lower clinical severity (Higgs *et al.*, 1982; Embury *et al.*, 1982). In the present investigations, SCA patients with α^+ -thalassaemia showed significantly higher mean red cell count (TRBC), lower MCV and lower MCH but no difference in mean hemoglobin levels.

Earlier reports on α^+ -thalassaemia in SCD patients in India have also shown significantly higher levels of hemoglobin, hematocrit (HCT), TRBC counts, HbA₂ levels and lower MCV, MCH resulting in milder clinical presentation (Mukherjee *et al.*, 1997b; Mukherjee *et al.*, 1998). Presence of alpha thalassaemia in sickle cell disease individuals results in reduction in intracellular concentration of HbS and consequent HbS polymerization and associated crisis (Steinberg *et al.*, 2012). In contrast, the MCV, MCH and MCHC indices of alpha thalassaemic SBT individuals were marginally higher but statistically insignificant.

The IVS1-5(G→C) mutation is the most common mutation found in India and the mutations included in the study are often responsible for β^0 -thalassaemia (Sinha *et al.*, 2009; Thein 2013). The prevalence of common beta thalassaemia mutations in different categories of population ethnic or otherwise will facilitate molecular pre and post natal testing to aid in clinical diagnosis in both symptomatic and non symptomatic individuals. The small sample size in the present studies may be considered as a bias or drawback but it illustrates wide

prevalence of the genetic diseases in the communities of the region emphasizing the need for broader studies. Finally the present study overwhelmingly demonstrates that more than one third sickle cell disease individuals in Madhya Pradesh carry the α -deletions. These coexisting mutations may not only modulate the hematological severity but also the clinical expression and serve as a predictor of disease severity.

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