

Influence of alpha-thalassemia -3.7kb mutation ($\alpha\alpha/-\alpha$ and $-\alpha/-\alpha$) upon clinical outcome of homozygous Sickle Cell Disease.

Abstract

Alpha thalassemia (α -thalassemia), fetal haemoglobin (HbF) and beta-globin haplotype are considered classical genetic disease modifiers in Sickle cell disease (SCD) causing clinical heterogeneity. Nevertheless their impact on clinical emergence and disease progression is still elusive. In this retrospective study, we have estimated the correlation of deletional α -thalassemia and associated factors like fetal haemoglobin (HbF) in patients with homozygous SCD from Odisha and Chhatisgarh, two of the central-eastern states of India. Six different types of deletional α -thalassemia were studied by Molecular analysis using gap PCR (N=267) and HbF% by Cationic exchange High performance Liquid Chromatography (CE-HPLC). Haematological, biochemical and radiological investigations were used to distinguish associated complications in the patients along with an account of malarial infections. Out of the total, 25.3% (n=68) SCD patients had deletional α -thalassemia presented better clinical profiles and haematological indices. Decrease in painful crisis (x2-12.5, $p<0.05$), chronic renal failure (CRF) along with priapism and leg ulcer were observed in alpha-thalassemia -3.7-kb mutation ($\alpha\alpha/-\alpha$ and $-\alpha/-\alpha$) in comparison to control SCD group. Females with single α - gene deletions ($\alpha\alpha/-3.7\alpha$) had significantly raised HbF% than corresponding males with a better clinical status and less medical consultations or hospitalizations. Thus, elevated level of HbF and presence of α -thalassemia mutation were well correlated with better sickle-cell RBC rheology, indicating an overall improved clinical picture of homozygous SCD.

Introduction: Alpha Thalassemia (α -thal.) and fetal haemoglobin (HbF) are well-known genetic modulators of homozygous and compound heterozygotes of Sickle cell Disease (SCD) *i.e.* sickle beta thalassemia (SBT) and HbSD hemoglobinopathies¹⁻⁴. Frequencies of α -thal in many tropical regions be attributed as a selection against malaria, however, its deviation from 4-gene status ($\alpha\alpha/\alpha\alpha$) imposes inconsistent medical deliberation in linkage. Both the said modulators are of high scientific priority research in the same context^{5,6}. Nevertheless, genetic variability of α -thal.in such endemic population is worthwhile in cohorts from discrete racial contextual.

Alpha Thalassemia (α -thal.) is one of the world's most common haemoglobin (Hb) disorder associated with deletion or, point mutation in α -globin gene cluster. Basically, it is characterized by microcytic anemia. The complex effects of the presence of α -thal. in patients with SCD may be result of two conflicting factors such as (i) reduced polymerization of HbS to less membrane damage, fewer dehydrated and irreversibly sickled cells and improved red cells survival and (ii) higher Hb concentration leading to increased blood viscosity. The two α - globin gene on chromosome 16 are located within 4Kb duplicated region leading to the possibilities of rearrangement including deletion & triplication with many related downstream consequences. A normal α -globin genotype can be represented as $\alpha \alpha / \alpha \alpha$. When both α -globin genes on a chromosome are deleted or otherwise inactivated, the condition is called α^0 thalassemia; because there is no output of α globin from the affected chromosome. When one of the linked α genes is inactivated, the condition is called α^+ thalassemia. In α^0 thalassemia there is no output of α globin and in α^+ thalassemia there is some output but usually only the product of a single α -globin locus.⁷

Presently, the available research data on the population frequencies that accounts for various form of α -thal is still scarce owing to the difficulties in the clinical screening of α -thal. phenotypes. Approximately 5% of the World's population carries globin gene mutation, of which 1.7% exhibit symptoms of α -Thal.⁸. In Africa, $\alpha 3.7$ del has outstanding prevalence ranging from 8-40% and it covers Bantu speaking Africa. Other countries like Australia, China, Cyprus, Egypt, Saudi-Arabia, Taiwan, Thailand has comparatively less prevalence of α -thal. than Africa that is ranging from 3-10%. India and Melanesia had highest cumulative frequencies of 50% including maximum alpha 3.7 & 4.2 del. respectively.⁹

Fetal Haemoglobin is known to linked with Arab-Indian haplotype strongly; inhibits sickling, by not participating in polymerization, and by diluting the concentration of HbS (sickle Hb) inside RBCs. It is still hailed as the first modifiers of SCD severity according to the classical observation long back (Watson 1948). The effect of HbF on the pathophysiology of SCD and HbS polymerization is well understood.¹⁰

In this work we intend to examine the correlation of deletional α -thal. with HbF.in patients with homozygous SCD from Odisha and Chhatisgarh, the two high burden central-eastern states of India known for thalassemias and hemoglobinopathies. The cumulative ameliorating factors were investigated using relevant haematological indices and clinical complications in the patients.

Materials & Methods

1. Study site, patient Population: Selected sample population belong to the central eastern states of India *i.e.* Odisha and Chhattisgarh – also known for their high malaria endemicity. The study was undertaken at the Sickle Cell Clinic and Molecular Biology Laboratory, V.S.S. Medical College Hospital (VSSMCH currently known as VIMSAR), Burla, Odisha, India and Department of Biotechnology, Centre For Advanced Life Sciences, Deogiri College, Aurangabad, Maharashtra.

2. Ethical approval patient enrollments and Follow-up: The study was approved by the Institutional Ethics Committee (IEC), VSSMCH. The Study subjects consisted of (a) patients attending the clinic with symptoms of SCD, (b) patients who were referred from other hospitals and (c) those found positive during cross-sectional prevalence studies for SCD (for 4 years). The patients were advised to come for a routine follow-up at the Sickle Cell Clinic at 3 months interval or earlier if they develop any health problem warranting medical attention. They were given a health diary to document any medical problem and treatment received outside. Patients who did not turn up for the scheduled follow-ups were contacted by personal visit of the Social worker of the Sickle Cell Clinic. During each visit patients underwent a detailed physical and laboratory examinations. Sickle cell positive samples in screening were subjected to High performance Liquid Chromatography (HPLC) analysis. For all study subjects, a detailed history was obtained including the current illness, previous state of health, family history and developmental history. Subjects of age ≤ 5 yrs and ≥ 60 yrs, those under active hydroxy-urea treatment and having taken blood transfusion (within less than 3 months) were excluded from enrollment. Clinical complications were evaluated and analyzed [supplementary data^{1, 10 & 11}].

3. Hematological Investigations: Hematological, biochemical investigations, radiological investigations and molecular analysis of all the patients were carried out to establish the relationship between the clinical (phenotypic) expressions with the underlying genetic factors. A venous blood sample (5ml) was collected in EDTA containing tubes after informed consent was obtained for each subject (signed by parents and guardians if age ≤ 18 years). RBC indices were measured in an automated blood cell counter (Sysmex K-1000, Kobe, Japan). Quantification of Hb variants (HbA, HbA₂, HbS, HbF) was done using an automated Hb-variant testing system using β -thal. Short Programme (BioRad, Hercules, CA, USA).¹²

4. Molecular genetic studies: Alpha Thalassemia determination was studied using Gap PCR method to identify the deletion break points. According to known DNA sequences around the break points, specific oligonucleotide primers were designed and PCR reactions were performed for single gene deletion (-alpha 3.7, -alpha 4.2), South-Asian double gene deletion (-SEA, -FIL) and Mediterranean double gene deletions (-alpha 20.5, MED) using a modified Multiplex PCR program. All the amplified PCR products were analysed using the same method as described above along with positive controls (kindly provided by Prof. S.S. Chong, McKusick-Nathans Institute of Genetic Medicine and Department of Pediatrics, Johns Hopkins School of Medicine, Baltimore, Maryland, USA on request).^{13,14}

5. Statistical analysis: Statistical tests of significance (Student's T and one-way ANOVA) were performed using Graph Pad Prism 8.0.

Results:

I. Age and sex distribution of α -thal. in SCD: Out of the total 300 SCD cases studied for β -globin cluster haplotype, 267 cases were amplified successfully for α -globin gene. Amongst them, 74.5% cases (n=199, Male/Female=127/72) had a normal α genotype ($\alpha\alpha/\alpha\alpha$), whereas 25.5% cases (n=68, Male/Female= 43/25) had α -thal. SCD with α -thal (62%) were having age ranging from 5-45 yrs (mean age 23.6 ± 10.3 yrs.). It was observed that highest number of patients were from the age group (21-30) yrs in both SCD with (38.2%) and without α -thal (38.6%) followed by 32.3% and 34.1% respectively in the age group (11-20) yrs. 76.4% (n=52) of the α -thal. cases were from non-tribal and rest 20.5% (n=14) were from Scheduled caste followed by 2.9% (n=2) from Scheduled tribes.

II. Association of deletional α -thal. with haemato-clinical indices of SCD: All the study groups shared close pathophysiology *i.e.* microcytosis. Worthwhile to mention here, that the association of Iron deficiency anemia (by quantitation of serum Iron, Ferritin and TIBC) and β -Thalassemia (Molecular Diagnosis & family study) was excluded from this study due to logistical constraints. A very decent effect was observed which included significantly lower MCV, MCH and higher RBC count ($p < 0.05$) as compared to SCD ($\alpha\alpha/\alpha\alpha$) (Table: 1). Associated clinical complications were curtailed except a very few. On comparison of different genotypes of α -thal. it was detected that 2-gene deletion ($-\alpha 3.7/-\alpha 3.7$) has better clinical features comparatively. The commonest clinical complications *i.e.* frequency of repeated painful crisis (VOC>2ep) was comparatively more in SCD ($\alpha\alpha/\alpha\alpha$) (χ^2 -12.5 &

p<0.05) than in SCD with α -thal along with frequency of hospitalizations.) There was no frequency of chronic renal failure (CRF), Priapism, leg ulcer in SCD with (- α 3.7/- α 3.7). However, avascular necrosis (AVN) and cholelithiasis were seen more in SCD α -thal. subjects, male & female respectively. The protective effect of deletional α -thal. on repeated episodes malaria was too observed (23.6% vs 11.2%) (Table: 2).

IV. Cumulative effect of α -thal and HbF on SCD females: Our analysis of female gender bias in the distribution of α -thal ($\alpha\alpha$ /-3.7 α , N=57) indicated that females with ($\alpha\alpha$ /-3.7 α) had significant raised HbF levels (p< 0.05) and decrease HbS% than males (p< 0.05) with a better clinical status except frequency of cholelithiasis and anaemia related issues. (Table :3&4).

Discussions:

An estimated 7 million babies are born each year either with a congenital abnormality or, a genetic disease, of which 90% belong to low or, middle income countries like India. A breakdown of the estimated annual births of the major Hb disorder observed that the major frequency of the severe forms of α -thal. is restricted mainly to southeast Asia.^[15] Deletional α -thal. is a common blood genetic disorder throughout the globe. In India alone, it has an average prevalence of 13% and the commonest is 3.7 Kb deletions. On the other hand, India is also a major harbour to Sickle cell disease (SCD) the highly variable clinical expressions of which, is influenced by no. of genetic and environmental factors^[16]. Under deoxygenated conditions, HbS polymerizes within the red blood cell (RBC), causing the RBC to be denser and less deformable, leading to increase in blood viscosity. Blood viscosity is affected by red cell aggregation impacted by reduced red cell deformability. HbS polymerization mediated through hypoxia and enhanced viscosity leads to vaso-occlusion and haemolytic anemia- central players of the pathophysiology of SCD, they precipitate a cascade of pathologic event, which in turn lead to a wide range of complications^{17, 18}. Moreover, the geographical interception of the prevalence of SCD, deletional α -thal. has scarcely been looked together in support for the malaria hypothesis in India. The present study has explored the possible correlation of deletional α -thal. with HbF in patients with homozygous SCD from two high burden central-eastern states of India – Odisha and Chattisgarh, also known malaria endemicity.

Earlier, several research groups have reported controversial findings about the protective effect of α -thalassemia. The protective effects of α -thalassemia on painful crisis, cholecystitis and epistatic factors like malarial infection and better haematological indices is

well documented. The patients used to experience fewer hemolysis associated complications, such as stroke, leg ulcers, pulmonary hypertension, and priapism¹⁹⁻²². In this investigation, we too have noted the better effect of less MCV and MCH upon a number of clinical events except leg ulcer, transfusion dependent anemia. Renoux et al. in one subset of population demonstrated higher rates of VOCs in sickle cell patients with homozygous alpha-thalassemia²⁴. However, another subset of data from a group failed to show a difference in blood viscosity at high shear rates as compared to patients without alpha-globin deletions.²³ These are different from our findings, where we have documented the controlling effect on repeated painful crisis along with associated hospitalizations. However, inter-individual variations in clinical presentations were found to be associated with sex, non-genetic factors and socioeconomic status along with deformability of RBC and viscosity. Amongst different α -globin genotypes, 2 α gene deletions were associated with better clinical presentation, absence of CRF, leg ulcer and priapism along with distinct haematological indices comparable to earlier published reports²¹⁻²⁵. Contrasting observations also documented in α -thal. deletion which was associated with decent risk of complications such as leg ulcer and kidney dysfunctions such as acute renal failure and chronic renal failure too.

Frequency of patients with HbF > 20% in the SCD (α -thal.) group appeared as a competent influencer which were associated with better clinical indices. In-vitro studies suggest that 20% HbF levels is necessary to prevent polymerization of HbS and it is the threshold level needed to prevent acute clinical events and prevent organ damage.^{26,27} Thus, elevated levels of HbF% improved sickle RBC rheology, which, in turn resulted in an overall better clinical picture of SCD in our subset of population which harboured deletional α -thal. mutations. Yet again, the sex based HbF increment need to be analyzed more in therapeutic context.

In summary, the findings of this study has clearly shown that SCD patients with deletional α -thal. presented with better clinical profiles and haematological indices. Reduced frequencies of painful crisis, no chronic renal failure (CRF), priapism and leg ulcer were noted in male patients, whereas females featuring $\alpha\alpha$ -3.7 α had significantly raised HbF% and decreased HbS% supporting better clinical status, and less medical consultations or hospitalizations against males. Nevertheless, cholelithiasis and infections were comparatively higher in female group which were with anemia- associated, owing to short life-span of rigid RBCs in comparison to same age-matched males. The chronic hemolysis caused by this condition often causes the formation of gallstones that can migrate and block the common bile duct leading to acute abdomen. Although the HbF% is comparatively higher, it didn't

show any beneficial effect upon the defined set of clinical events characteristics of SCD. The protective effect of deletional α -thal. in SCD patients having repeated episodes of malaria was significantly evident. Female gender bias was indicated in the frequency distribution of α -thal. mutations with significantly higher HbF% levels and lower HbS% in females than males, and an overall better clinical status.

According to reports from WHO, at least 30% of the World population is α -thal carrier. Thus, it should be considered in the differential diagnosis of hypochromic, microcytic anaemia specially to rule out iron-deficiency anaemia and β -thalassemias in clinically suspected and geographically relevant communities. Prenatal screening of α -thal. should be advocated along with genetic counselling of high-risk couples of SCD for prenatal diagnosis, for susceptible caste/ tribe sowing to it's the silent nature of α -thal.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

Table-1: Prevalence of Deletional α -Thalassemia in SCD

α-Globin Gene Status in SCD	No. of Cases (%) (N=267)
normal α genotype ($\alpha\alpha/\alpha\alpha$)	199 (75%)
3.7 heterozygous α -thal ($\alpha\alpha/-\alpha3.7$)	57(21%)
4.2 heterozygous α -thal ($\alpha\alpha/-\alpha4.2$)	3(1%)
3.7 homozygous α -thal. ($-\alpha3.7/-\alpha3.7$)	8(3%)

Table-2: Comparison of Hematological features among SCD without and with α -thal.

Parameter	SCD ($\alpha\alpha/\alpha\alpha$) n=199	SCD ($\alpha\alpha/-\alpha3.7$) n=57	SCD ($-\alpha3.7/-\alpha3.7$) n=8	SCD ($\alpha\alpha/-\alpha4.2$) n=3	F	P-Value
Hb(g/dl)	8.4 \pm 2.2	8.9 \pm 2.2	8.2 \pm 2.5	8.7 \pm 1.6	0.8	NS
RBC$\times 10^6$/cumm	3.09 \pm 1.01	3.56 \pm 1.19	3.51 \pm 1.12	4.02 \pm 0.19	3.8	0.01
HCT (%)	25.7 \pm 7.7	27.9 \pm 8.3	26.1 \pm 9.7	29.1 \pm 7.0	0.9	NS
MCV (fl)	84.5 \pm 10.2	78.7 \pm 10.8	74.5 \pm 5.2	72 \pm 15.7	30	0.001
MCH (pg)	28.3 \pm 5.03	25.9 \pm 4.9	24.08 \pm 3.1	21.7 \pm 3.1	5.23	0.01
MCHC(g/dl)	33.2 \pm 5.1	32.8 \pm 4.1	32.2 \pm 4.6	30.4 \pm 3.4	0.3	NS
HbA₂ (%)	2.4 \pm 0.9	2.7 \pm 0.9	2.6 \pm 0.8	2.9 \pm 0.7	1.79	NS
HbF (%)	22.3 \pm 7.0	21.9 \pm 8.7	17.3 \pm 5.0	14.9 \pm 10.7	0.5	NS
HbS (%)	72.4 \pm 7.0	71.6 \pm 7.3	74.88 \pm 7.18	80.9 \pm 10.0	0.68	NS

*One way Anova test

Table-3: Comparison of Clinical Features among SCD without and with α -thal.:

Clinical Parameters	SCD without α-thal ($\alpha\alpha/\alpha\alpha$) N=199	SCD with α-thal N=68
Incidence of Painful Crisis	164 (86%)	57 (83.8%)
Hospitalization for VOC	108(54%)	23(34%)
VOC > 2ep/Yr	54(27%)	5(8%)
Transfusion Dependent Anemia	115(58%)	39(57.3%)
Splenomegaly	97(49%)	37(55%)
Splenic Atrophy	29(14%)	5 (7.3%)
Hepatomegaly	76(38%)	25(36.7%)
Repeated episodes of Malaria (>1)	47(23.6%)	8(11.7%)
PTB	13(6.5%)	2(2.9%)
Jaundice	46(23.1%)	9(13.2%)
AVN	18(9%)	4(5.8%)
Cholelithiasis	37(18.5%)	6(9.4%)
CRF	5(2.5%)	1(1.4%)
Leg Ulcer	5(2.5%)	2 (2.9%)
Priapism	2(1%)	0(0%)

Table-4: Comparison of Clinical Features among SCD without and with different α -genotypes:

Clinical Features	SCD ($\alpha\alpha/\alpha\alpha$) n=199	SCD ($\alpha\alpha/-\alpha3.7$) n=57	SCD ($-\alpha3.7/-\alpha3.7$) n=8	SCD ($\alpha\alpha/-\alpha4.2$) n=3	χ^2	p-Value
Incidence of Painful crisis	164(86%)	46(80%)	8(100%)	3(100%)	2.4	0.4
Frequency of Painful crisis (VOC/Pt/yr)	1.08 \pm 1.2	1.05 \pm 1.2	1.08 \pm 0.9	1.6 \pm 1.0	NS	NS
Hospitalisations for Painful crisis	103(54%)	30(52%)	5(71%)	2(67%)	0.6	0.89
VOC>2Ep	51(27%)	4(8%)	1(14%)	1(33%)	12.35	0.006
Transfusion dependent Anemia	115(58%)	30(53%)	7(88%)	2(67%)	3.6	0.3
Frequency of Blood transfusion (BT/Pt/Yr)	0.2 \pm 0.5	0.1 \pm 0.4	0.1 \pm 0.1	0.05 \pm 0.06	NS	NS
History of Total Hospitalisations	144(72.3%)	37(64.9%)	7(87.5%)	2(67%)	NS	NS
Frequency of Hospitalisations (Hospitalisations/Pt/Yr)	0.2 \pm 0.5	0.1 \pm 0.1	0.1 \pm 0.1	0.3 \pm 0.3	NS	NS
Infections	85(43%)	24(43%)	3(43%)	1(33%)	0.1	0.9
Splenomegaly	97 (49%)	30 (52%)	3(38%)	2(67%)	6.5	0.16
Splenic Atrophy	28(14%)	2 (3%)	2 (25%)	1(33%)	6.5	0.16
Hepatomegaly	76 (38%)	22(38.5%)	3(37.5%)	0(0%)	1.8	0.6
Avascular necrosis	18 (9%)	2(3%)	1(12.5%)	1(33%)	4.5	0.2
Cholelithiasis	37(18.5%)	9(15.7%)	1 (12.5%)	0(0%)	1.04	0.7
Chronic Renal Failure	5 (2.5%)	1(1.7%)	0(0%)	0(0%)	0.5	0.9

Leg Ulcer	5(2.5%)	2(3.5%)	0(0%)	0(0%)	0.4	0.9
Priapism	2(1%)	0(0%)	0(0%)	0(0%)	0.6	0.8

*Chi square test **One-way Anova test, # NS- Not Significant

Table:5

Sex wise distribution of Alpha thalassemia ($\alpha\alpha$ -3.7 α) and its probable

Table:5.1 Comparison of abnormal Hb%

Abnormal Hb%	Male (N=37)	Female (N=20)	p-Value
HbA	2.32±0.5 (3.7-1.4)	2.13±0.24 (3.2-0.0)	0.24
HbA ₂	2.8±0.8 (4.6-0.8)	2.45±0.9	0.07
HbS	73.7±7.67(87.6-54.5)	60.5±5.6 (83.0-69.8)	0.046
HbF	20.71±8.3 (40.9-5.8)	25.52±5.8 (35.0-10.10)	0.02

*Unpaired t-test

Table:5.2 Comparison of Clinical profiles

Clinical Complications	Male (N=37)	Female (N=20)
History of Hospitalization	27 (72.9%)	10(50%)
Avg. rate of hospitalizations	2.35	1.5
Bone Problem	02(5%)	0 (0%)
Splenomegaly	19 (51.3%)	13 (65%)
Hepatomegaly	15 (40.5%)	07(35%)
Multiple Infections	02 (5%)	02(10%)

Episodes of Malaria	18(48.6%)	7(35%)
Cholelithiasis	02(5%)	07(35%)
Leg Ulcer	02 (5%)	0 (0%)