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Clinico-Pathological analysis of Hb S/β+ Th Patients in Odisha: A Tertiary Care Hospital Based Study

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Abstract

India is ethnically a diverse country with marked regional variation. Due to migration, there is constant mixing of people from different regions. These migrations not only helped in creating variations leading to positive mutations but also caused many genetic abnormalities leading to inheritance of genetic disorders. The most common genetic disorder that occurs during the neonatal period is hemolytic anemia. Being multifactorial in nature, this disease is predominantly intrinsic to erythrocytic dysfunction. These dysfunctions are related to structural and functional abnormality of erythrocytes, thus leading to hemoglobinopathies. From clinical as well as epidemiological point of view, prevalency within hemoglobinopathic mutations are sickle cell anemia and thalassemia. These mutations affect population with origin in Africa, the Mediterranean region, Southeast Asia, the Middle East and the Far East. Around 1-2% of the global population is heterozygous for Hb S and 3% are heterozygous for β-thalassemia. Sickle cell disease (SCD) and thalassemia are the most common forms of hereditary hemolytic anemia. Apart from monogenic inheritance, co-inheritance is also seen leading to compoung heterozygosity within the population. Persons with Hb S/β- thalassemia major are almost never symptomatic at birth because of the presence of Hb F, but symptoms begin to develop by six months of age. They often die of cardiac complications of iron overload by 30 years of age. Beginning transfusion and chelation therapy are difficult challenges for parents to face early in their child's life. Therefore, this pilot study was attempted to observe the prevalence of compound heterozygosity within a particular population. If a bone marrow transplant is a possibility, the blood for transfusion should be negative for cytomegalovirus. Hematopoietic stem cell transplantation has cured >1,000 patients who have S-β-thalassemia major. We think that antenatal screening or screening of higher secondary school children to detect hemoglobinopathies, counselling of the individuals with hemoglobinopathies is expected to help in drastically reducing the incidence of the disease. We need to reduce the burden of genetic disease by implementing programs such as population screening, genetic counselling and prenatal diagnosis. The available data for Hb S/β- thal are mostly based on the patients attending hospitals for treatments and their immediate family members. This data will help in planning population screening programs and thus, consequently result in the reduction of genetic diseases.

Keywords: Hb S/β-Thal; Hemoglobinopathies; Transfusion.

Introduction

Human Hb is a globular tetramer formed by the combination of two "type α" (α or ζ) polypeptide (globin chains) with two "type β" (β, δ, Gγ, Aγ or ε) chains. Each chain has its own prosthetic heme group, which forms a reversible bond with the oxygen molecule (O_2), thereby fulfilling the primary function of Hb, which is to transport O_2 from the lungs to peripheral tissues (Old, 2007). Synthesis of each of the globins is controlled by

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distinct genes, which are arranged in two clusters; the genes that code for the α and ζ chains (α clusters) are located in the telomeric region of the short arm of chromosome 16 (16p 13.3), whereas the genes that code the β, δ, γ and ε chains (β *cluster*) are on the short arm of chromosome 11 (11p 15.5). During the embryonic period, the embryonic Hb variants Gower I (ζ2ε2), Gower II (α2ε2) and Portland I (ζ2γ2) are produced; during the fetal period these are substituted by fetal Hb or Hb F (α2γ2), which then cedes its place to Hb A (α2β2) and A_2 (α2δ2) in adulthood (Dhaliwal, 2004) (Fig. 1). Six months after birth, Hb A predominates absolutely, making up more than 95% of total cellular Hb, while Hb A_2 levels are around 2-3%, and Hb F levels are 0-2% (Catlin, 2003).

Hemoglobinopathies are the result of mutations that affect the globin genes and can be classified into two major groups namely

a) Structural alterations, which form anomalous Hb variants, and

b) synthesis alterations (thalassemia), where one or more types of globin chain are partially or completely suppressed. Less frequently, the two phenotypes can occur in combination. Less frequently the two phenotypes can occur in combination.

Structural hemoglobinopathies are generally caused by simple substitutions, small insertions or deletions of bases that affect coding regions of the genes and lead to amino acids in the protein chain being substituted (Khattab *et al*., 2006).

Notable among these is Hb S ($\alpha_2\beta S_2$), a variant that affects position 6 on the β chain, substituting Glutamic acid with Valine (β6 Glu→Val). It was described by Itano and Pauling (1949) as a form of Hb found in the red blood cells of patients with sickle-cell anemia (SCA), with electrophoretic migration that differentiated them from normal individuals. When Hb S is in its deoxygenated state (deoxy-HbS) and in elevated concentrations, it polymerizes, resulting in abnormally rigid and inflexible red blood cells (sickle red blood cells). These in turn lead to chronic hemolysis and vaso occlusion, , which are the pathophysiologic bases of the disease (Shah, 2004).

The thalassemia is the result of a reduction, or an absence of production, of one or more globin chain types, leading to a buildup of another type, the synthesis of which is unaffected. The excess chains are unstable and precipitate, leading to changes to the erythrocyte membrane and early destruction of the cell (Thein, 2004). Furthermore, the deficient hemoglobinization of erythrocytes results in hypochromia and microcytosis, which are characteristic abnormalities of this group of diseases. Thalassemia are classified as α, β, γ, δ, δβ or γδβ, depending on the type of chain whose production is affected. Thalassemia α and β are the most common, while the majority of the first are caused by deletions that remove α genes (Tolentino and Friedman, 2007). The β-thalassemias are generally the result of substitutions of bases on the exons, introns and promoter regions of β genes (Vekilov, 2007). It should be noted that if both the traits are inherited together, then this compound heterozygotic condition is denoted by HbS-β-thalassemia. However, co-inheritance of Sickle cell anemia and β- thalassemia (HbS-β thal) is mainly determined by β thal genes variants (Tefferi, 2004; Shah, 2004; Madigan and Malik, 2006).

During the process of mutation, LCR plays a vital role. In case of humans, LCR is physically defined by 5 HS region (Hypersensitivity region) which is responsible for transcription stimulation at high levels (Fig 1). Individual HS region have different roles in the remodeling of chromatin as well as globin gene switching and they are as follows:

i. HS_1 – It is located to the 5′ end of ε-globin gene and this region show no sign of hematological defect.
ii. HS_2 - It is a general enhancer element. It encodes for E-box sequences which forms the binding sites for the basic helix-loop-helix proteins such as USF and Tal1.
iii. HS_3 - It is a general weak enhancer element. It helps in chromatin remodeling.
iv. HS_4 - It helps in chromatin remodeling.

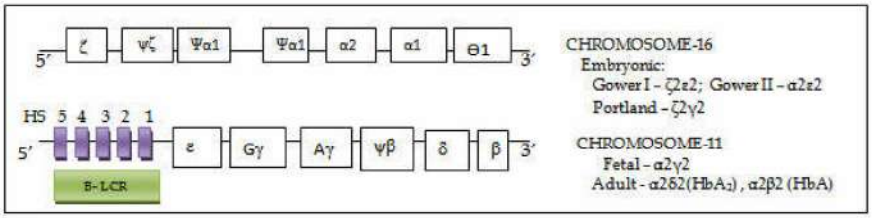

Fig. 1: Clusters of α and β-globin genes with β-Locus Control Region

v. HS_5 - It is a constitutive hypersensitive site which acts an insulator.

The abnormalities in the synthesis of Hb chain, both qualitatively and quantitatively can be assessed presently by using a number of conventional methods, such as HbF estimation, HbA2 estimation, CBC and so on (Urbinati et al., 2006).

Table 1: Variants in sickle β- thalassemia patients

Sl No	Variants of S-β-T	Efficacy
1	Mild Hb S/β Th	It is observed in about 15% of all cases in Southeast Asia. This group of patients maintains Hb levels between 9 and 12 g/dl and usually does not develop clinically significant problems. No treatment is required.
2	Moderately Hb S/β Th	The majority of HbE/beta-thalassemia cases fall into this category. The Hb levels remain at 6-7 g/dl and the clinical symptoms are similar to thalassemia intermedia. Transfusions are not required unless infections precipitate further anemia. Iron overload may occur.
3	Severe Hb S/β Th	The Hb level can be as low as 4-5 g/dl. Patients in this group manifest symptoms similar to thalassemia major and are treated as thalassemia major patients.

Methods and Methodology

A) Population study

While investigating the etiopathogenesis of 30 subjects with anemia, we came across with 10 subjects with Hb S/β Th. Herewith, we report the clinical findings of 10 patients with HbS received from tertiary care hospital. Present study relates to the results of clinical and hematological examination of 10 patients with HbS. Male to female ratio was 1:1. Six of 10 patients belonged to Balasore district; three other patients were from Banki, Nayagarh and Keonjhar of Odisha, India.

Age: Above 2 years and below 30 years.

Inclusion Criteria: Patients having high HbF, low/absence of HbA and not having more than 8 transfusions per year.

Exclusion Criteria: Transfusion cases of 21days, patients having any associated chronic illness.

B) Methodology

Venous blood samples were collected and smears were prepared. Blood amounting 2.5 ml was collected in a tube containing EDTA as an anticoagulant. It was mixed well and analyzed. Smears were stained by Leishman's stain and examined. Total hemoglobin was estimated by Sahli's method. Sickle cell test was also undertaken. It is a slide based test for sickling using sodium metasulphite. CBC and HPLC were followed using automatic cell counter

a) Cohort study (through clinical reports and questionnaire) was undertaken to analyze any symptomatic parameter (Vichinsky *et al.,* 2005; Wenning and Sonati, 2007; Wintrobe and Foerster, 2004).

b) PBS was carried out to study the morphology of blood cells. To prepare a PBS, 0.2 gm of powdered Leishman's dye was added to 100 ml of methanol (acetone free) and the mixture was warmed at 50^0C in the shaking waterbath for 15 min. The solution was then filtered and allowed to stand at room temperature for 24 h before use (Jison *et al.,* 2004; Vekilov, 2007).

c) Solubility test for HbS was performed following the standardised protocol. Sickling confirmatory test was performed by using 2% sodium metabisulfite solution (Itano and Pauling, 1949; Hustman et al., 1970; Vichinsky et al., 2005). Nestroft screening was followed to screen β-thal heterozygotes.

d) HPLC was undertaken by using Variant™ Hb testing system, Biorad.

C) Key parameters to be analysed in CBC

Structural hemoglobinopathies may have an impact on the red cell indices, and red cell indices are critical to the diagnosis of thalassemias. The key components of the CBC include Hb, red blood cell (RBC) number, mean corpuscular volume (MCV), and red cell distribution width (RDW) (Tefferi, 2004). The procedures were standardized by following the standardized protocol (Wintrobe and Foerster, 2004).

The **RDW** is a measure of the degree of variation in red cell size. Some causes of microcytic anemia, most notably iron deficiency, are characterized by an increase in RDW (Steiner *et al.*, 2007). Therefore, the RDW may provide information useful as an adjunct to diagnosis but is not useful as a lone indicator.The normal RDW level is 10.2 to 14.5%.

The **RBC count** is also useful as a diagnostic adjunct because the S-β-thalassemias produce a microcytic anemia with an associated increase in the RBC number. Other causes of microcytic anemia, including iron deficiency and anemia of

chronic disease, are more typically associated with a decrease in the RBC number that is proportional to the degree of decrease in Hb concentration.

Mean corpuscular volume (MCV) is the average volume of red cells in a specimen. MCV is elevated or decreased in accordance with average red cell size, i.e., low MCV indicates microcytic (small average RBC size), normal MCV indicates normocytic (normal average RBC size), and high MCV indicates macrocytic (large average RBC size). The reference range for MCV is 80-96 fL/red cell in adult.

The **Hb concentration** typically is decreased in S-β-thalassemia.

TThe **hematocrit** measures the volume of red blood cells compared to the total blood volume (red blood cells and plasma). The normal hematocrit for men is 40 to 54% whereas for women it is 36 to 48%. This value can be determined directly by microhematocrit centrifugation or calculated indirectly.

The **mean corpuscular hemoglobin** (**MCH**), or "mean cell hemoglobin" (**MCH**), is the average mass of hemoglobin per red blood cell in a sample of blood. It is reported as part of a standard complete blood count. **MCH** value is diminished in hypochromic anemias. MCH levels are between 27 and 31pg.

Mean cell hemoglobin concentration (MCHC) is the average concentration of hemoglobin in a given volume of blood. Normal MCH levels are between 27 and 36 g/dl for adults and between 32 and 34% for children. If the MCHC level is below 28%, this is considered to be too low. The MCHC level can be too low because of blood loss overtime, too little iron in the body, or hypochromic anemia.

Observation and Discussion

From the above study, following aspects have been observed:

a. The morphological picture of PBS in case of HbS-β thal patients is nearly similar to β-thal homozygotes showing moderate to severe aniso-poikilocytosis, hypochromia, microcytosis, polychromatic red cells and occasional target cells along with nucleated normoblasts. The presence of nucleated RBCs (NRBCs), polychromasia, sickle cells, target cells, Howell-Jolly bodies, and Pappenheimer bodies were also observed. Neutrophilia and thrombocytosis may also be observed (Fig. 2).

b. Sickling test shows the presence of sickling cells. Solubility test for the presence of HbS shows a positive turbidity resulting in visual impairment of the bold dark lines,thus, confirming the presence of HbS. NESTROFT test shows the presence of a partial visibility, thus, showing partial positivity (-/+) and confirming the presence of β-thalassemia heterozygotes (Table 3).

c. There is a significant decrease in MCV for all groups (26 fl - SS, 28.8 fl - S/Beta0 thalassemia and 20.8 fl - S/Beta+ thalassemia). Microcytosis resulting from the action of HU in S/Beta thalassemia patients measured by MCV, is lower than that is observed in homozygous S at six months of treatment but it reaches a comparative value after more than one year. Microcytosis and hypochromia due to the thalassemia mutation contribute to this effect (Table 2).

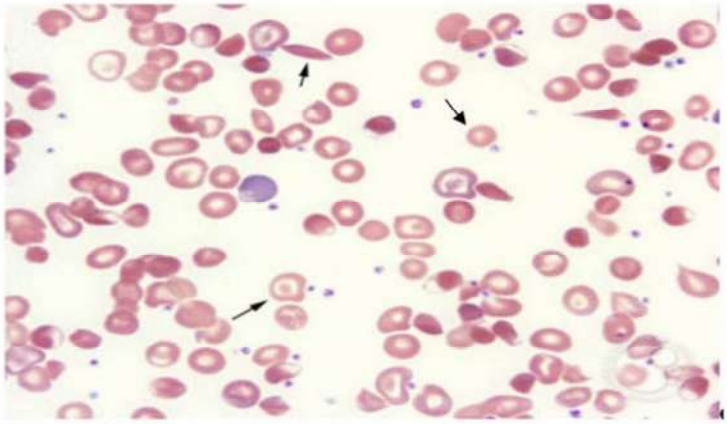

Fig. 2: Peripheral blood smear

d. The MCH concentration does not reduce significantly, but it is slightly higher in S/beta thalassemia. S/ß0 thalassemia results from the absence of ß-chain production that causes red blood cell (RBC) instability due to excess α chains, leading to abnormal erythropoiesis. S/ß0 thalassemia can be differentiated from sickle cell anemia based on RBC morphologic characteristics. S/ß0 thalassemia is characterized by microcytic, hypochromic RBCs, along with the presence of target cells and fewer sickle cells (Table 2).

e. The expected hemoglobin electrophoresis results in blood specimens from patients with sickle cell anemia show the following values: 80% sickle cell hemoglobin (HbSS), 1% to 20% hemoglobin F (HbF), 2% to 4.5% hemoglobin A2 (HbA2), and absence of hemoglobin A (HbA) if the patient has not recently received a transfusion. These cases had a large range of variation in hemoglobin level (5.2-13.0%), but the majority had moderate to severe anemia (Table 3).

Table 2: Various indices of clinical components

Case No	Subjects	Age (in yrs)	Sex	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)	WBC ($x10^3$/µl)	RBC ($x10^6$/µl)
01	Father	37	M	13.7	45	68	28	28	7.8	11.6	5.9
	Mother	25	F	10.6	34	56	22	33	8.9	9.4	5.0
	Son	06	M	10.9	28.2	22	18.0	30	18.3	17.9	3.7
02	Father	29	M	12.7	40	70	28	27	8.4	8.2	4.5
	Mother	21	F	10.5	36	65	27	28	8.1	7.5	4.0
	Son	04	M	12.2	29.3	28.8	17.5	29	15.1	14.4	3.5
03	Father	37	M	13.9	45	65	31	35	9.5	9.5	4.6
	Mother	23	F	09.5	40	69	25	29	8.2	8.7	3.8
	Son	07	M	11.2	30.7	20.8	22.1	25	15.5	15.7	3.3
04	Father	41	M	13.9	50	80	30	36	9.7	11.4	5.0
	Mother	31	F	10.6	44	75	25	29	10.5	9.5	3.8
	Daughter	10	F	11.5	27.9	32.3	20.0	29	16.7	14.6	2.9
05	Father	33	M	14.1	51	65	29	36	11.4	10.5	4.8
	Mother	26	F	11.5	40	75	27	27	8.6	8.7	4.1
	Son	06	M	10.7	26.0	20.8	16.8	29	17.5	14.6	3.1
06	Father	29	M	13.8	55	71	30	32	9.5	9.5	4.5
	Mother	24	F	11.5	36	62	27	29	10.2	8.5	3.9
	Daughter	05	F	09.5	31.4	20.8	23.2	25	15.8	13.7	2.9
07	Father	34	M	12.6	51	80	31	36	8.5	10.7	5.0
	Mother	26	F	10.8	37	75	25	32	10.2	8.5	3.8
	Daughter	06	F	10.2	30.6	29.9	21.2	26	16.5	11.6	3.1
08	Father	38	M	14.2	50	65	31	29	10.4	11.7	4.9
	Mother	25	F	11.2	41	70	29	27	9.5	9.4	4.1
	Son	04	M	12.3	32.4	27.9	20.2	29	15.9	12.4	3.2
09	Father	42	M	13.7	49	80	29	35	9.5	11.5	5.0
	Mother	37	F	10.5	36	73	27	30	9.7	9.5	4.4
	Daughter	10	F	10.5	33.2	35.3	24.3	29	16.9	14.5	3.1
10	Father	30	M	13.5	55	91	28	30	10.4	10.5	4.9
	Mother	27	F	10.5	40	85	26	29	11.7	7.3	4.1
	Daughter	05	F	09.7	30.4	32.6	22.2	27	17.6	11.5	2.9

Table 3: HPLC data of 10 patients

Case No.	Subjects	Age (in yrs)	HbF	HbA	HbA_2	HbS	Sickling %
01	Son	06	23.9	2.8	2.9	61.3	Positive
02	Son	04	22.7	14.4	2.5	76.5	Positive
03	Son	07	17.8	7.3	2.4	69.5	Positive
04	Daughter	10	19.4	11.5	3.4	63.3	Positive
05	Son	06	17.6	8.8	2.5	69.0	Positive
06	Daughter	05	18.0	2.8	2.8	0	Negative
07	Daughter	06	28.9	2.4	2.7	65.3	Positive
08	Son	04	18.5	2.6	2.6	0	Negative
09	Daughter	10	23.7	2.2	2.4	63.2	Positive
10	Daughter	05	22.9	2.4	2.5	0	Negative

It is apparent that the majority of the sickle cell-β- thalassemia cases showed reduced values of red cell indices like HCT, MCV, MCH and MCHC manifesting hematological aberrations before blood transfusion (Table 2). The RBC values were either reduced or normal in the above 10 cases of sickle cell-β-thalassemia. HPLC also showed a predominant fluctuation in HbF, HbA_2, HbS and HbA values. Our study also projects the sickling percentage in the above subjects. Based on the above results, the effectiveness of screening can be compared with the clinical cases present previously for better knowledge and understanding. We also suggest that genetic counseling and prenatal diagnosis can be used for reducing the burden of increasing genetic disease.

Conclusion

Differentiation of sickle cell anemia, beta thalassemia and the sickle beta thalassemia syndromes need to be undertaken carefully due to close similarity of symptoms and laboratory findings, i.e., microcytosis, hypochromia, target cells and sickle cells in the peripheral smear. The hemoglobin electrophoresis pattern of the sickle-beta thalassemia consists of high HbS with an increase in HbF, HbA2 and low HbA value. The present study highlights the coinheritance of β-thalassemia and Hb S gene, which is wide spread in southern and western Odisha. Further it is assumed that a large number of such double heterozygote cases remain undiagnosed or misdiagnosed leading to premature death without proper treatment. Molecular diagnosis of Hb D, HbE or Hb S gene is required along with characterization of β-globin gene mutations in this region. The prenatal diagnostic facilities and services, genetic/ marriage counselling are the ultimate aims to be achieved. This is a preliminary study and we will carry out the beta globin gene mutation to establish the above facts in more detail.

Ethical Clearance

This study confirms the ethical principles of Medical research developed by World Medical Association, Declaration by Helsinki (1999). Ethical clearance was given by the Institutional Ethical Committee (IEC) of SCB Medical College and Hospital, Cuttack, Odisha to work in the Department of Clinical Hematology and Department of Pediatrics, SCB Medical College and Hospital, Cuttack. Vide letter number IEC/IRB No.-824/11.03.2019..

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Conflict of Interest: None

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