

## Structural Hemoglobin Variants: Mutation, Hematology and Its Application in Prenatal Diagnosis

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**KEY WORDS** Hemoglobin variants; prenatal diagnosis; mutation; polymerase chain reaction; thalassemia; hematology.

**ABSTRACT** Referred cases of Genetics OPD, SGPGI between 1988-2000 were screened for cause of anemia. Out of which one hundred and ninety eight individuals were found as abnormal hemoglobin variants and associated with  $\beta$ -thalassemia mutations viz: E $\beta$ T, S $\beta$ T, D $\beta$ T. These phenotypes were of severe type. Hematology and mutation analysis were performed in these subjects by ARMS-PCR technique. Due to high prevalence of IVS 1-5 (G-C) mutation, most of the structural variants (69%) were found associated with this mutation. Severity of the thalassemia syndromes (E $\beta$ T, S $\beta$ T, D $\beta$ T) emphasized the need of establishment of prenatal diagnosis for common structural hemoglobin variants along with beta thalassemia mutations.

### INTRODUCTION

Hemoglobinopathies in India are important public health problems. Of the several abnormal hemoglobin molecules, there are three variants- Sickle Cell (HbS), hemoglobin E (HbE) and hemoglobin D (HbD) that are predominantly prevalent in India. Though there are regional variations of these different types of hemoglobinopathies in different parts of India, the cumulative gene frequencies have been found to be 5.35% (Balgir 1996). The average gene frequency of Sickle Cell and hemoglobin D in India has been observed 4.3% & 0.86% respectively and Hb E in North Eastern region is 10.9% by Balgir (1996). An effort has been made to perform hematology in different type of hemoglobinopathies and mutation analysis by ARMS-PCR technique has also been performed as part of establishment of antenatal diagnosis for hemoglobinopathies.

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### MATERIAL AND METHODS

One hundred and ninety eight individuals referred from Genetics OPD between 1988-2000 for investigation of cause of anemia have been included in this study. The index cases and available family members were clinically evaluated and investigated for the cause of anemia.

Red Blood Cell Indices were measured on automated Sysmex 800. Osmotic fragility was calculated according to the test described by Dacie and Lewis (1991). Fetal hemoglobin (HbF) was estimated by alkali denaturation technique reported by Betke et al. (1959). Quantitative estimation of hemoglobin A2 (HbA2) was carried out by micro-column chromatography described by Schleider et al. (1977). Sickling test was performed by the method of Dacie and Lewis (1991). Prepared hemolysates were subjected to Hb electrophoresis on cello gel at pH 8.5 in tris-glycin buffer for 90 mins. DNA extracted from peripheral blood leucocytes by phenol chloroform method described by Poncz et al. (1962). ARMS-PCR technique by Newton et al. (1988) was applied for characterization of the point mutations of  $\beta$ -globin gene. To confirm the presence of Hb D, Hb E and Hb S mutations allele specific amplification (ASA) primers listed in table 1 were used. The structures of primers described by Varawalla et al. (1991) were used for characterization of the  $\beta$ -thalassemia mutations.

### RESULTS

Of the 198 individuals, 78 were found to be associated with Hb E disease (39.4%), 90 with Hb S disease (45.5%) and 30 with Hb D disease (15.1%). Of the 90 individuals with Hb S disease, 25 had sickle cell disease, 55 were Sickle cell trait and in 10 cases Hb S was associated

**Table 1: Structure of primers**

Hb variant	Primers used	Fragment size (bp)	Sequences of primers 5'----->3'
S (A->T) [β6(A3)Glu->Val]	Common Primer ASAPrimer Mutant ASAPrimer Normal	197bp	ACC TCA CCC TGT GGA GCC A AGT AAC GGC AGA CTT CAC CA AGT AAC GGC AGA CTT CAC CT
E (G->A) [β26(B8)Glu->Lys]	Common Primer ASAPrimer Mutant ASAPrimer Normal	254bp	ACC TCA CCC TGT GGA GCC A AAC CTG CCC AGG GGC CTT AAC CTG CCC AGG GGC CTA
D [β121(GH4)Glu-Gln] Loses Eco RI site	Direct Primer Reverse Primer	861bp* 552bp & 309bp	CAA TGT ATC ATG CCT CTT TGC ACC GAG TCA AGG CTG AGA GAT GCA GGA

\* Absence of site

with β-thalassemia mutation. Out of 78 cases of Hb E disease, it was found to homozygous Hb E disease, Hb E trait and Hb E associated with β-thalassemia in 4, 27 and 47 cases respectively. Hb D carrier status was found in 20 individuals where as in 10 cases it was associated with β-thalassemia mutation.

The hematological parameters are summarized in table 2. Eβ-thalassemia is associated with severe anemia with Hb of 6.1±1.6. There is no fall of Hb in E-trait but mild anemia is seen in Hb E disease. Microcytosis and hypochromia is seen in both Hb E Homozygous and in Eβ-thalassemia. Osmotic fragility at 0.36% normal saline seems not to be a reliable indicator as the SD ranges from 18-20%. Hb F is raised significantly

in Eβ-thalassemia. Hb E level in homozygous was found to be 81.4±1.6 % where as in Eβ-thalassemia it was 50.4±16.6 % & in E trait was 28.7±6.3%.

The difference between the percentage of abnormal Hb E in E trait and in Eβ-thalassemia was significant with higher value found in the latter.

Sickle Cell disease and Sβ-thalassemia seems to have moderate to severe anemia with mean Hb of 7.6g%. Microcytosis was associated with Sβ-thalassemia but not with Sickle Cell disease or S trait. Osmotic fragility at 0.36% of normal saline seems not to be a reliable parameter with SD ranges between 22-29%. Hb F is raised significantly in Sickle cell disease and Sβ-thalassemia and minimally with S trait. Percentage of

**Table 2: Hematological profile of structural hemoglobin variant patients**

(a)							
Type	Hb g%	MCV fl	MCH pg	O.F. %	Hb A2%	HbF %	Abnormal Hb%
Eb-T (47)	6.1±1.6	67.7±8.2	20.0±3.1	56.0±18.7	-	21.6±10.8	50.4±16.6
Sb-T (10)	7.3±1.9	69.1±8.0	23.5±3.2	44.0±22.1	4.5±0.5	14.9±6.4	59.9±5.8
Db-T (10)	11.2±2.1	65.7±12.7	24.0±5.9	53.7±23.5	5.7±2.4	1.5±0.9	55.3±25.8
(b)							
Type	Hb g%	MCV fl	MCH pg	O.F. %	Hb A2%	HbF %	Abnormal Hb%
E trait (27)	11.6±2.7	78.7±10.6	27.4±4.5	71.7±17.9	-	1.2±1.3	28.7±6.3
S trait (55)	10.9±2.6	80.5±12.2	27.2±6.0	53.2±28.6	2.6±0.6	2.1±3.5	36.1±12.0
D trait (20)	10.4±2.2	79.3±14.5	27.6±6.3	83.4±19.9	2.6±0.4	1.3±1.5	33.1±9.3
(c)							
Type	Hb g%	MCV fl	MCH pg	O.F. %	Hb A2%	HbF %	Abnormal Hb%
EE (4)	9.2±1.1	62.8±6.9	21.1±3.5	62.5±20.2	-	2.4±1.9	81.4±1.6
SS (25)	7.9±2.0	82.2±10.9	26.3±3.7	49.6±23.4	2.5±0.6	13.5±6.0	67.0±9.7
DD	-	-	-	-	-	-	-

Hb S does not differentiate Sickle Cell disease from S $\beta$ -thalassemia, though S trait has lower value than the other two. Hb A2 is increased in all cases of S $\beta$ -thalassemia only.

Similar to Hb E structural variant HbD disease affects the hematological profile (low MCV and low MCH) irrespective of its association with  $\beta$ -thalassemia mutations. Osmotic fragility at 0.36% of normal saline is reduced (<80%) in all cases of D $\beta$ -thalassemia and D trait with a wide range of variation (19-23%). Quantitation of Hb F may not be helpful in differentiating D trait from D $\beta$ -thalassemia rather percentage levels of HbD and HbA2 is found to be significantly raised in D $\beta$ -thalassemia.

Mutation analysis confirmed the presence of Hbs, S, E and D in respective samples. The IVS I-5 (G-C) mutation was found in 46; 619 bp deletion in 5; Codon 15 in 4; Frameshift mutation 41/42 (-CTTT) in 4; Codon 16 in 2 and Codon 30 (G-C) in 3 subjects.

### DISCUSSION

The hematological heterogeneity among structural hemoglobin variants associated with  $\beta$ -thalassemia is quite evident from the table. Subjects of hemoglobin variants associated with  $\beta$ -thalassemia mutations are severe anemic with microcytic and hypochromic picture and are very similar to homozygous  $\beta$ -thalassemia while subjects with milder anemia are normocytic and normochromic. High Hb F values are known to reduce the clinical severity of the disease. In our patients, however, the HbF levels were not significantly different in E $\beta$ T and S $\beta$ T groups. However, the high levels of Hb F in Sickle Cell disease group confirm the presence of milder phenotypes of Sickle Cell disease with low Hb levels. This phenotype in association with  $\beta$ -thalassemia mutation further enhanced the clinical severity of the disease like HbE disease. Hence the factor responsible for milder phenotype in Sickle cell disease may be different from the protective values of HbF. The abnormal Hb accounts 50-60% of the total Hb in all the subjects where structural variants are associated with  $\beta$ -thalassemia mutations (E $\beta$ T, S $\beta$ T and D $\beta$ T).

The distribution of variant hemoglobin in India has been related to various ethnic groups.

$\beta$ -Thalassemia is the most frequent monogenic disorder in the country. Due to the practice of non-random mating pattern, specific ethnic group has few specific common mutations. It is seen to be associated with other abnormal Hb and may lead to a more severe phenotype.

As observed in this study its association with Hb E and Hb S leads to severe anemia but not with HbD. Osmotic fragility is not a good parameter to use except for D $\beta$ -thalassemia where it is reduced. Percentage of abnormal HbE differentiates homozygous and E $\beta$ T from heterozygous E. There is no significant difference between the amounts of HbS in Sickle Cell disease and S $\beta$ -thalassemia though the mean amount in Sickle cell disease is higher than S $\beta$ -thalassemia. HbD can have wide variation in D trait and with associated  $\beta$ -thalassemia.

Of the 90 cases of Sickle cell disease and S $\beta$ -thalassemia, 54 subjects are of U.P. origin. To our knowledge, there is no previous reports of HbS from U.P. Knowing the clinical severity of HbS, if in homozygous state or associated with  $\beta$ -thalassemia comes it warrants antenatal diagnosis for carrier couples. So to diagnose the simple sickling test may be included in routine hematology lab for screening of hemoglobinopathies. Agarwal et al. (1997) reported 19 families of E $\beta$ -thalassemia of Uttar Pradesh (U.P.) origin and emphasized the utility of antenatal diagnosis for thalassemia syndromes. The commonest  $\beta$ -thalassemia mutation in U.P. is IVS I-5 (G-C) reported by Agarwal et al (2000). This paper is in concordance of the previous observation thus the maximum association (69%) of IVS I-5 (G-C) mutation was found with structural hemoglobin variants. Due to high prevalence rate and phenotypic severity of beta thalassemia alone and associated with structural hemoglobin variants there is need for establishment of prenatal diagnostic facilities for hemoglobinopathies. However, to know the prevalence of the hemoglobin variants needs a large population based study.

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