HAEMATOGENETICS

Prevalence and Molecular Characterization of Red Cell Pyruvate Kinase (PK) Deficiency.

Year of commencement : 2000

Year of completion : 2005

18 cases of neonatal jaundice and 66 cases of non-spherocytic hemolytic anemia were investigated for pyruvate kinase deficiency by assaying the enzyme activity spectrophotometrically. Three cases showed a deficiency of pyruvate kinase activity (1.58, 4.6 and 4.75 IU/g Hb respectively) with a compensatory rise by 2 to 3 fold in 2,3, DPG levels and some reduction in ATP levels. The remaining cases showed normal PK activity (Range 8.28 to 19.40 IU/ g Hb). The hematological and biochemical parameters are shown in Table 1. One case of PK deficiency (case 1) had a history of neonatal jaundice, had received an exchange transfusion at that time and subsequently he received 30 transfusions up to the age of 10 yrs. He had undergone splenectomy one year back and another case with hemolytic anemia aged 32 yrs had been transfused six months prior to investigations.

Genomic DNA were extracted from the PK deficient patients by a standard protocol (QIAGEN MINI Kit). Amplification of the genomic DNA was performed using exon specific primers. All exons of the *PK-LR* gene that code for the R-type isoenzyme were screened by single-strand conformation polymorphism (SSCP) analysis in all three patients. Each exon was amplified by polymerase chain reaction (PCR) using 500 ng genomic DNA. Altered mobility on SSCP analysis of exon 9, 10 and 11 of the PKLR gene in the 3 propositi confirmed the presence of a mutation in these regions (Figure 1A-C). The mutations were confirmed by sequence analysis using both the forward and reverse primer on a DNA sequencer (ABI prism 310, Applied Biosystem). 4 different mutations were detected among the 3 PK deficient patients (1436G→A, 1190A→T, 1456C→T, 1291G→A, (Table-2 and Figure-2 A-C). Case 1 showed the presence of the 1436 G→A homozygous mutation in exon 10 and both the parents were heterozygous for the same mutation. Case 2 was a compound heterozygote showing two mutations 1456 (C→T) and 1190A→T mutation by sequencing and Case 3 showed the

1291 G→A identified by DNA sequencing of exon 9 while the second mutation is not yet characterized.

Table 1: Hematological and biochemical analysis in PK deficiency

	Case 1 (UN)	**Case 2 (TS)***	**Case 3 (JP)***
Age/sex	10yrs/M	32yrs/M	28yrs/F
Hb (g/dl)	7.5	14.4	12.4
RBC (10^6 X mn^3)	2.16	4.65	3.94
HCT (%)	23.0	42.0	36.4
MCV (fl)	106.5	90.3	92.4
MCH (pg)	34.7	31.0	31.5
MCHC(%)	32.6	34.3	34.1
Retic (%)	18.84	1.5	1.0
Bilirubin Total (mg%)	3.3	NA	NA
Indirect (mg%)	2.8	NA	NA
G6PD (IU/mlRBC/min)	8.5	6.8	7.2
PK (IU/gHb)	1.58	4.6	4.75
2,3-DPG (μmols/gHb)	21.8	18.4	19.5
ATP (μmols/gHb)	3.4	2.9	3.1

* Post Transfused

Normal ranges

G6PD - 2.5 – 5.5 1u/mlRBC/min

PK (Neonates) - 7.2 – 12.5 1u/gHb

PK (Adults) - 11.5 – 16.5 1u/gHb

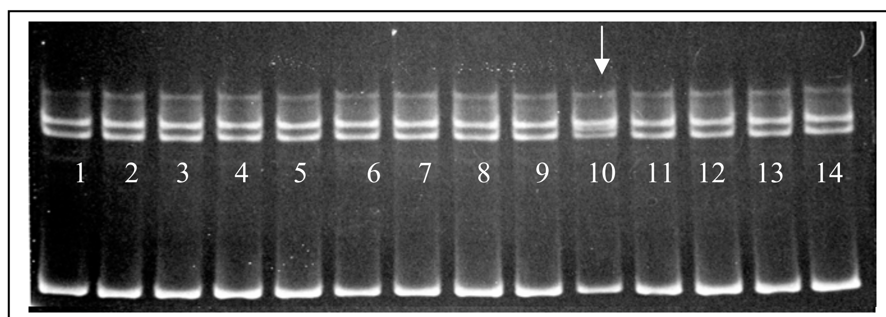
2,3 DPG - 8.16 – 14.7 μmols/gHb

ATP - 3.22 – 5.2 μmols/gHb

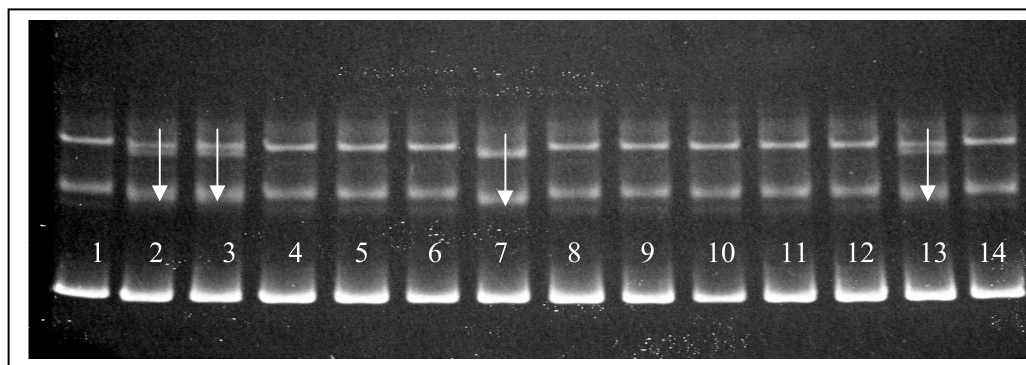
Table-2 : Molecular data of PK deficient patients

	SSCP Altered mobility	Mutation	Amino acid substitution
Case 1	Exon 10	1436G→A (Homo)	479 Arg→His
Case 2	Exon 9 and 11	1190 A→T/ 1456C→T	407 Glu→Lys and 486 Arg→Trp
Case 3	Exon 10	1219G→A/?	431 Ala→Thr/ ?

Fig-1 A] PCR-SSCP in Exon 9 Patient No. 2 (Lane no. 10) showed altered mobility



B] PCR-SSCP in Exon 10 in case no. 1 (Lane no.7) showed altered mobility.



C] PCR-SSCP in Exon 11 in case 3 (Lane no. 1, 11& 12) showed altered mobility.

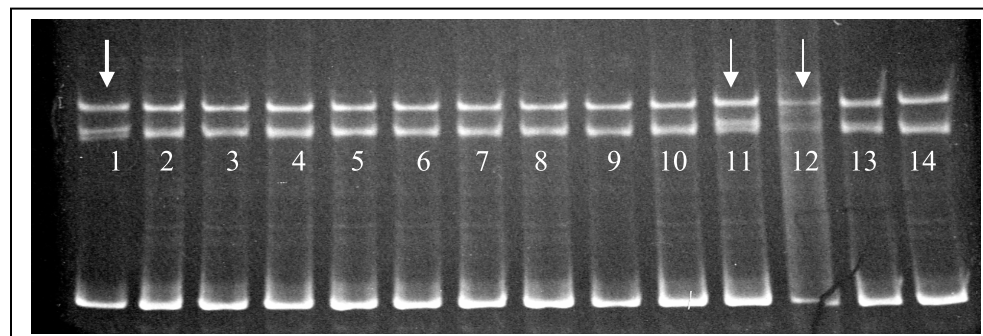


Fig. 2A: Case 1 showed the presence of 1436G→A Homozygous mutation

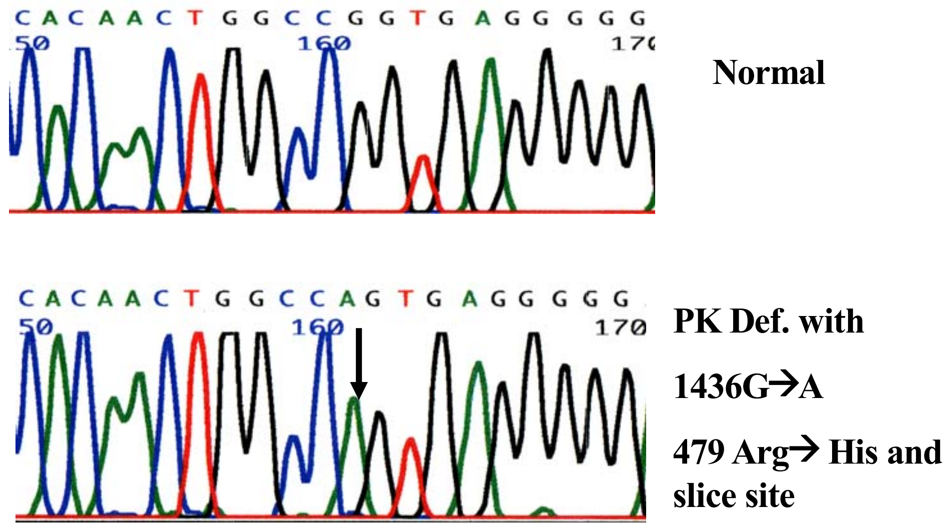


Fig. 4B Case 2 was a compound heterozygote showing two mutation 1456C→T and 1190A→T.

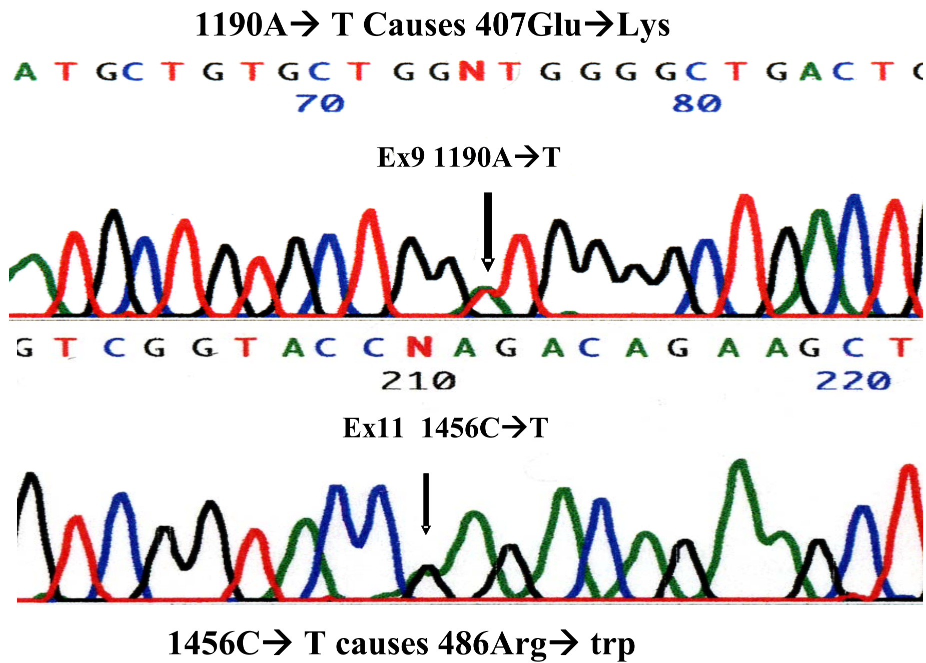
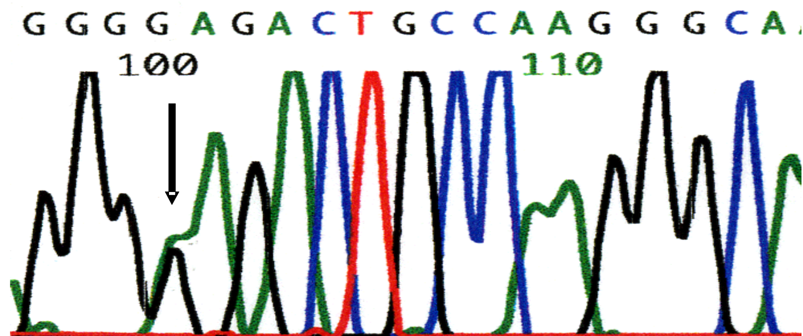


Fig. 4C: Case 3 showed heterozygous mutation at 1219G→A



1291G→A causes 431Ala→Thr

Glucose-6-phosphate dehydrogenase mutations and haplotypes in various ethnic groups of India.

Year of Commencement: ***2004.***

Year of Completion: ***2007.***

The Indian population comprises of numerous caste and tribal groups, each with characteristic physical, cultural, and genetic background. Since India is endemic for malaria, G6PD deficiency is found in most of the populations. Considering the diversity of the Indian population, it is highly probable that many variants of G6PD deficiency may exist in the Indian sub-continent. However, there is little information about the mutations causing G6PD deficiency and their distribution among the diverse Indian population. The aims and objectives of the present study are:

1. To characterize the mutations underlying G6PD deficiency.
2. To see the distribution of these mutations in different population groups of India.
3. Study different polymorphic sites in the G6PD gene (Haplotype) to define the genetic frameworks as well as the origin of different mutations.

A total of 36 G6PD deficient individuals from the tribal groups of Gujarat and Maharashtra were screened for mutational analysis. Initially all the samples were screened for G6PD Orissa (131C→G) mutation in exon 3 and 4 by PCR-RFLP analysis. When this mutation was found to be absent, the samples were screened for G6PD Mediterranean (563C→T) and G6PD Kerala Kalyan (949G→A) mutations in exon 6 & 7 and 9 respectively. Table 1 shows the number of G6PD deficient samples from Gujrat and Maharashtra and the distribution of G6PD mutations in different tribal groups.

In Gujrat, out of 29 G6PD deficient samples, 20 were from Kolcha tribe, 5 were from Kotwadia tribe and 4 were from Kathodi tribe of Valsad district. G6PD Mediterranean was found to be the major polymorphic variant in all the tribal groups with an overall prevalence of 68.9% followed by G6PD Kerala Kalyan (20.6%). None of the individuals showed the presence of G6PD Orissa mutation. Three individuals (10.3%) did not show the presence of any of the three mutations. Further characterization of these samples is in progrss.

In Maharashtra, 10 G6PD deficient individuals from the Katkari tribe of Raigad district were screened for mutational analysis. Four individuals (40%) were found to have G6PD Mediterranean mutation; whereas remaining 6 individuals did not show the presence of G6PD Orissa mutation. However, these 6 individuals are yet to be tested for G6PD Kerala Kalyan mutation.

As reported in the last year, in Maharashtra out of 32 G6PD deficient individuals, 19(63.3%) were found to be G6PD Orissa mutation, 3 (10%) were G6PD Kerala-Kalyan mutation and 10 remained uncharacterized. Similarly in Tamilnadu, out of 40 G6PD deficient individuals, 26 (70.2%) were found to have a very rare G6PD Namoru mutation in exon 3 and 4 whereas remaining 14 individuals did not show the presence of either G6PD Orissa or G6PD Mediterranean mutations. These individuals were further tested for G6PD Kerala-Kalyan mutation in exon 9 by PCR-RFLP and 4 (10.8%) of them showed the presence of G6PD Kerala-Kalyan mutation. Hence, 10 G6PD deficient individuals remained uncharacterized. DNA sequencing for exon 9 was carried out on the uncharacterized samples both from Maharashtra and Tamilnadu and none of the individuals showed the presence of any mutation in exon 9. Further sequencing for the other exons will be undertaken.

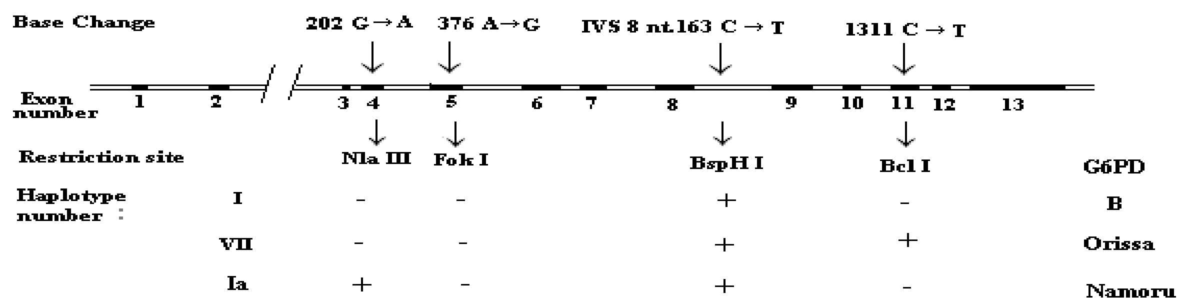
Four polymorphic sites of the G6PD gene namely NlaIII, FokI, BclI, BsphI have been standardized by PCR followed by restriction enzyme digestion for Haplotype analysis. Polymorphisms were recorded as (+) if the restriction site was present and as (-) if it was absent. Haplotypes associated with G6PD Orissa and G6PD Namoru mutations along with the normal controls (G6PD B) from the same population groups is shown in Fig.1. G6PD Orissa and G6PD Namoru mutations were mainly associated with haplotype # VII and Ia respectively whereas G6PD B was associated with haplotype I. Standardization of other two polymorphic sites (Pst1 in exon 10 and PvuII in intron 5) in the G6PD gene are in progress.

Table 1: Distribution of G6PD Mutations in different tribal groups.

State	District	Tribes	Total	G6PD Mediterranean (563C→T)	G6PD Orissa (131C→G)	G6PD Kerala Kalyan (949G→A)	Uncharacterised
Gujrat	Valsad	Kolcha Kotwadia Kathodi	20 5 4	17 (85.0%) 1 (20.0%) 2 (50.0%)	0 0 0	2 (10.0%) 2 (40.0%) 2 (50.0%)	1 (5.0%) 2 (40.0%) 0
		TOTAL	29	20 (68.9%)	0	6 (20.6%)	3 (10.3%)
Maharashtra	Raigad	Katkari	10	4 (40.00%)	0	NT	6 (60.0%)
		TOTAL	10	4 (40.00%)	0	NT	6 (60.0%)

NT: Not tested

Fig 1: Haplotypes of the G6PD gene in the tribals of Maharashtra and Tamilnadu.



Incidence and Molecular characterization of G6PD deficiency of North East India.

Year of commencement: ***2003.***

Year of Completion: ***2006.***

The aims and objectives of the study are as follows:

1. To determine the frequency of G6PD deficiency in North East India.
2. To characterize the mutations underlying G6PD deficiency.
3. To determine the genotype-phenotype correlation.

A total of 2986 blood samples from different ethnic groups of Assam and Mizoram were screened for G-6-PD deficiency by RMRC Dibrugarh. The study cohort comprised of 1262 males and 1724 females. The majority of the samples are the representatives of Assam and one hundred eighty samples of Mizoram. Screening for G6PD deficiency was done by DPIP dye decolourization method.

A total of 40 G6PD deficient samples (Males-30, Females-10) were received at IIH, Mumbai from RMRC Dibrugarh for mutation analysis. A strategy based upon sequential steps was used. DNA was amplified using PCR and was tested for the presence of the following common Indian mutations by restriction fragment length polymorphism (RFLP):

Mutation	Exons	Nucleotide Change	Restriction Enzyme	Fragment size (bp)	
				Normal	Mutant
G6PD Mediterranean	6 & 7	563 C→T	Mbo II	381,127,67	261,127,120,67
G6PD Orissa	3 & 4	131 C→G	Hae III	107,75,66,48,45,11	123,107,66,45,11
G6PD Kerala-Kalyan	9	949 G→A	Mnl I	74,60,54,21,18	80,60,21,18

The prevalence of G-6-PD deficiency in various ethnic groups of Assam and Mizoram is shown in Table1. The prevalence of G-6-PD deficiency varied from 0.5 to 9.5% in different ethnic groups with an overall prevalence rate of 2.9%. The prevalence of G6PD deficiency among the males and females was 4.4 % and 1.3 % respectively.

Table 1: Distribution of G6PD deficiency among various ethnic groups of Assam and Mizoram.

Caste/Ethnic groups	Total Tested	Total Deficient
Ahom	487	14 (2.8%)
Bihari	42	4 (9.5%)
Bodo	143	3 (2.1%)
Brahmin	189	1 (0.5%)
Chutia	38	2 (5.2%)
Kachari	141	8 (5.6%)
Kaibarta	128	4 (3.1%)
Kalita	472	11 (2.3%)
Kayastha	13	0
Keot	18	0
Koch	132	2 (1.5%)
Muslim	244	8 (3.3%)
Nath	24	1 (4.2%)
Rabha	108	3 (2.8%)
Rajbanshi	42	1 (2.4%)
Tea Garden Workers	159	11 (6.9%)
Others	426	11 (2.6%)
Mizo	180	5 (2.8%)
Total	2986	89 (2.9%)

Out of 40 G6PD deficient individuals, 16 were found to have G6PD Orissa (40.0%), 6 were G6PD Kerala-Kalyan (15.0%) and 3 were G6PD Mediterranean (7.5%) mutations. Hence, 15 (37.5%) individuals remained uncharacterized. To look for other mutations, DNA sequencing was done on 6 individuals for exons 3,4 and 9 however, no mutations were observed. Further sequencing of these samples for other exons will be undertaken.

Isolation of fetal cells and cell free fetal DNA from maternal blood for Non-Invasive Prenatal Diagnosis of Hemoglobinopathies

Year of commencement : 1999

Year of completion : Ongoing

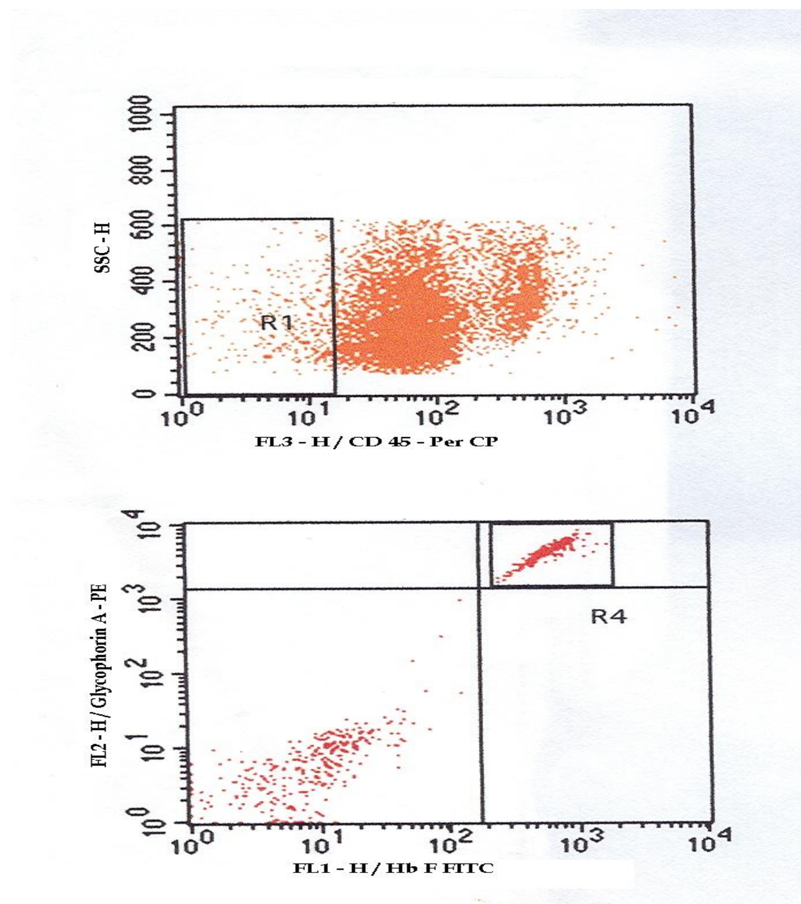
We have continued the work of isolating fetal cells from maternal blood and circulatory cell free DNA from maternal plasma.

FETAL NRBCs FROM MATERNAL BLOOD:

The isolation of NRBCs was continued with the modified protocol as described last year. We have modified the Fluorescence Activated Cell Sorting using the combination of 3 monoclonal antibodies CD45, Glycophorin A and Human fetal hemoglobin in a single sorting step.

The enrichment of the fetal cells was done by using a discontinuous Percoll density gradient. On enrichment the cells were permeabilized using formaldehyde and then incubated with CD45 perCP, Glycophorin A tagged with Phycoerythrin and anti fetal hemoglobin tagged with FITC. We used a single sorting strategy wherein first the CD45 negative cells were gated and then from those gated cells we sorted the dual positive cells which showed positive fluorescence for both Glycophorin A and Hb F (Fig 1). The percentage of the fetal cells at different periods of gestation ranged from 0.01% to 2.03%.

Fig 1: Two-dimensional bivariate histogram display from FACS. The 1st histogram displays the CD 45 negative population, which is gated (Gate R1). The 2nd histogram displays the dual positive population of Hb F –FITC and Glycophorin A – PE depicted in Gate R4.



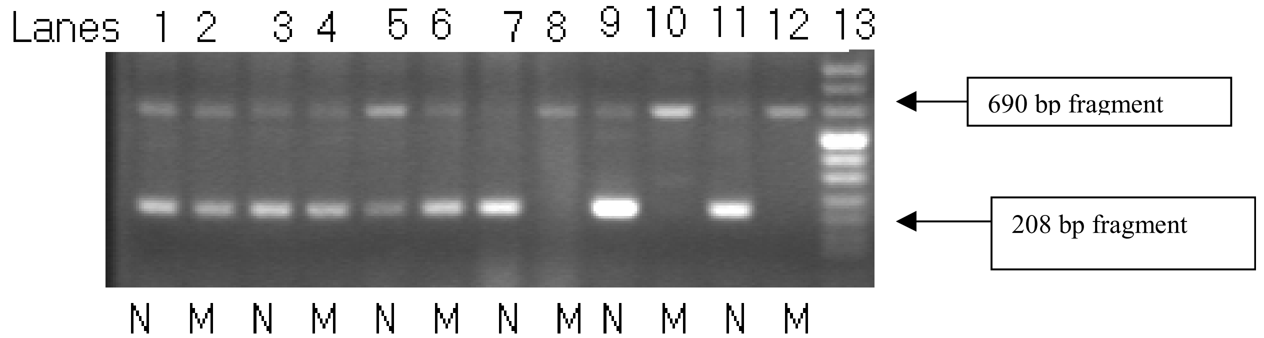
The DNA was isolated from the fetal cells by using the QIAMP Blood Kit (Qiagen) and used for genetic analysis. The mutations causing the hemoglobinopathies in the parents were first characterized. In 26 of the cases, we first amplified a 739 bp fragment, using 10 µl of fetal DNA. This was followed by a nested PCR using a 1: 100 dilution of the first PCR product. The nested PCR approach was based on ARMS and the primers were chosen on the basis of the mutation of the parents. These results were compared with those obtained by the invasive diagnostic procedures and are shown in Table 1. We were able to give accurate diagnosis in 20 out of the 27 cases (74.07 %). In 3 cases we got maternal contamination whereas in 4 cases we were not able to give any diagnosis due to non-specific amplification. Two of the results are depicted in Figs. 2 & 3.

Table1: Results of the DNA isolated from the fetal cells :

Key: * : Non-specific amplification # : Maternal contamination

Sr no	Mother's Mutation	Father's mutation	Mutation in the CVS	Mutation in the Fetal cells
1	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
2	Codon 5 heterozygous	Codon 5heterozygous	Normal	Normal
3	Hb S heterozygous	Hb S heterozygous	Normal	Normal
4	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
5	Codon 30 heterozygous	Hb S heterozygous	Hb S heterozygous	Hb S heterozygous
6	IVS 1-5 (G → C) heterozygous	Codon 41/42 heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
7	Codon 5 heterozygous	Codon 5 heterozygous	Codon 5 heterozygous	Codon 5 heterozygous
8	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
9	619 bp deletion	Codon 5 heterozygous	619 bp deletion	619 bp deletion
10	Hb S heterozygous	Hb S heterozygous	Hb S homozygous	Normal*
11	IVS 1-5 (G →C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
12	Frameshift 8/9 heterozygous	Frameshift 8/9 heterozygous	Frameshift 8/9 heterozygous	Normal*
13	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
14	Hb S heterozygous	Hb S heterozygous	Normal	Normal
15	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
16	Codon 41/42 heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
17	Uncharacterized	Frameshift 8/9 heterozygous	Frameshift 8/9 absent	Frameshift 8/9 absent
18	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	Normal	Normal
19	Hb S heterozygous	Hb S heterozygous	Normal	Hb S heterozygous#
20	Codon 41/42 heterozygous	Codon 41/42 heterozygous	Codon 41/42 heterozygous	Hb S trait*
21	Hb S heterozygous	Hb S heterozygous	Normal	Hb S trait#
22	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G →C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous
23	Codon 15 heterozygous	Codon 15 heterozygous	Normal	Codon 15 heterozygous#
24	Frameshift 8/9 heterozygous	619 bp deletion	Frameshift 8/9 heterozygous	Normal*
25	Hb S heterozygous	Hb S heterozygous	Normal	Normal
26	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) heterozygous	IVS 1-5 (G → C) homozygous	IVS 1-5 (G → C) homozygous
27	Codon 5 heterozygous	IVS 1-5 (G → C) heterozygous	Codon 5 heterozygous	Codon 5 heterozygous

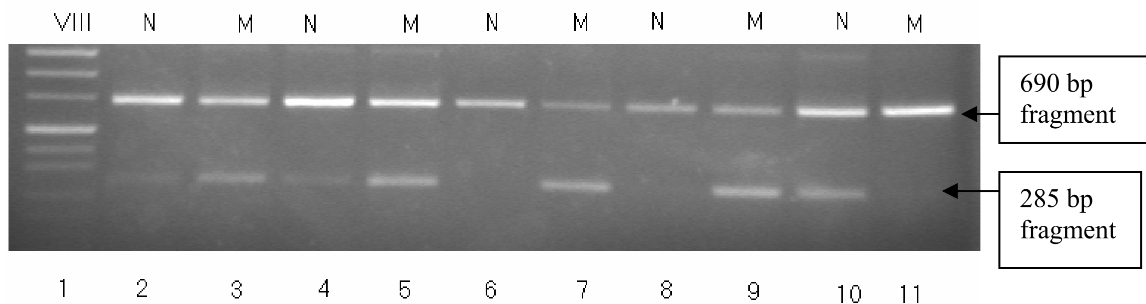
Fig 2: Agarose gel electrophoresis of the Nested ARMS PCR product in Case 25



Lanes:

1 & 2:	Mother	HbS heterozygous
3 & 4:	Father	HbS heterozygous
5 & 6:	1st child	HbS heterozygous
7 & 8:	CVS	Normal
9 & 10:	Fetal DNA	Normal
11 & 12:	Negative control	Normal
13:	Marker VII(Roche)	

Fig 3: Agarose gel electrophoresis of the Nested ARMS PCR product in Case 26

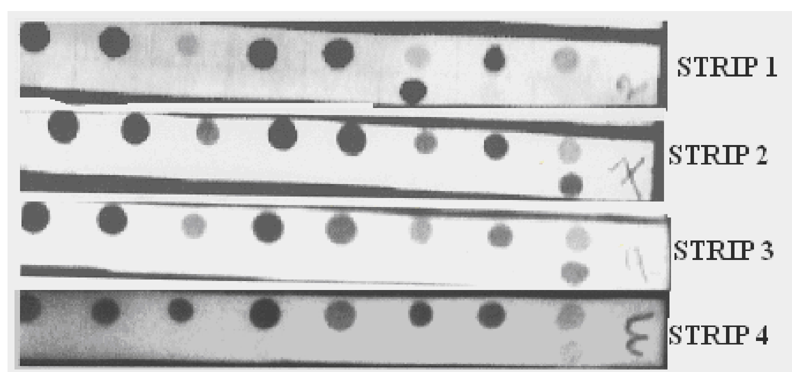


LANES:

1:	MARKER VIII (Roche)	
2 & 3:	Mother	IVS 1nt 5 (G–>C) heterozygous
4 & 5:	Father	IVS 1nt 5 (G–>C) heterozygous
6 & 7:	CVS	IVS 1nt 5 (G–>C) homozygous
8 & 9:	Fetal cells DNA	IVS 1nt 5 (G–>C) homozygous
10 & 11:	Negative control	IVS 1nt 5 (G–>C) absent

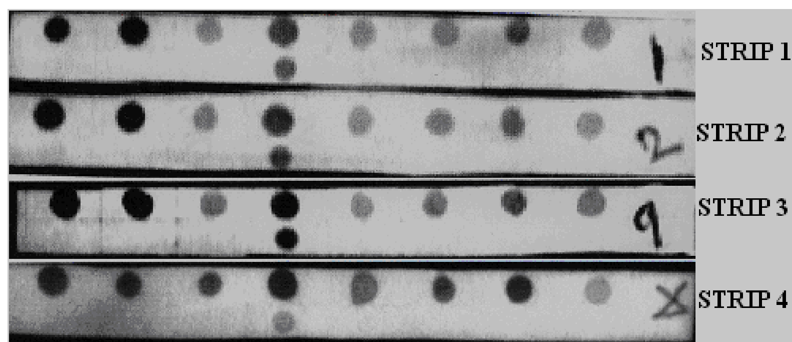
We also tried using the Covalent reverse dot blot hybridization method. In this method, 10 µl of fetal DNA was used first to amplify an 846bp fragment with primers external to the primers China 1 and PCO 6 used for CRDB. This was followed by a nested PCR using the primers China 1 and PCO 6 primers (739bp fragment). 10 µl of the PCR product was loaded on a 1% agarose gel to check for specific amplification and remaining 40 µl was used for hybridization. Although we were able to detect the mutation in the fetus by this method in 8 out of the 12 cases, we also got non-specific binding to the probes in 2 out of the 12 cases. 2 of the results are shown in figures 4 & 5.

Fig 4: Results of CRDB after Nested PCR in Case 5



Strip 1	Mother	Codon 30 heterozygous
Strip 2	Father	HbS heterozygous
Strip 3	CVS	Hb S heterozygous
Strip 4	Fetal cells DNA	Hb S heterozygous

Fig 5: Results of CRDB after Nested PCR in Case 13



Strip 1	Mother	IVS 1 nt 5 (G → C) heterozygous
Strip 2	Father	IVS 1 nt 5 (G → C) heterozygous
Strip 3	CVS	IVS 1 nt 5 (G → C) heterozygous
Strip 4	Fetal cells DNA	IVS 1 nt 5 (G → C) heterozygous

CIRCULATORY CELL FREE FETAL DNA

We have continued with the use of the nested PCR approach to identify the mutations using the circulatory cell free DNA isolated from the maternal plasma. The samples were chosen wherein the maternal and paternal mutations were different. The plasma was obtained by centrifuging the peripheral venous blood at 1600g for 10 minutes. This plasma was then recentrifuged at 16000g for 10 minutes. The cell free DNA was then extracted from the plasma using QIAMP Blood kit (Qiagen). We used a nested PCR approach similar to that used for fetal cells and were able to detect the presence of the paternal mutation in 3 cases (Table 2).

Table 2: The results of the fetal DNA isolated from the maternal DNA:

Sr. No.	Mother's Mutation	Father's Mutation	Mutation detected in the CVS	Mutation detected in the Plasma DNA
1	Codons 8/9 (+C) heterozygous	IVS 1 nt 1 (G→T) heterozygous	IVS 1 nt 1 (G→T) heterozygous	IVS 1 nt 1 (G→T) heterozygous
2	619 bp deletion heterozygous	IVS 1 nt 5 (G→C) heterozygous	IVS 1 nt 5 (G→C) heterozygous	Presence of IVS 1 nt 5 (G→ C) shown
3	IVS 1 nt 5 (G→C) heterozygous	Codon 16 (-C) heterozygous	IVS 1 nt 5 (G→C) + Codon 16 (-C)	Presence of Codon 16 (-C) shown

Molecular characterization of α thalassemia by Multiplex PCR

Year of commencement : 2005

Year of completion : Ongoing

α thalassemias are the commonest single gene disorders in the world. DNA analysis carried out in certain regions of India has shown that $-\alpha^{3.7}$ and $-\alpha^{4.2}$ are the commonest single gene deletions. Some larger deletions like --- 20.5kb , --- SA, ---- SEA have been reported in the Indian population. Earlier we were doing α genotyping by Southern blot hybridization. We have now established the multiplex PCR strategy for screening for α gene deletions.

Eight deletions causing α thalassemia ($-\alpha^{3.7}$, $-\alpha^{4..2}$ ---- SEA , THAI , --- SA, --- 20.5kb , -- med, ---FIL), were screened by using single tube multiplex PCR. The criteria used for selection of samples for screening α thalassemia were reduced red cell indices with increased RBC counts in relation to the Hb level and normal or reduced HbA_2 levels.

50 samples were screened for deletional α thalassemia which included 31 individuals of β - thalassemia trait and 19 HbS homozygotes / HbS – β thalassemia patients. 8 samples (10%) showed a single gene deletion and 3 (6%) showed the two gene deletional defect. $-\alpha^{3.7}$ deletion was the commonest defect encountered.

Molecular characterization of abnormal hemoglobins

We encountered 3 samples showing altered mobility on cellulose acetate electrophoresis and an abnormal peak on HPLC (Biorad Variant, Hb Testing system)

Hb Agenogi –(β 90 (F6) Glu → Lys)

Two samples showed an abnormal band (Hb X) moving near HbA_2 on cellulose acetate electrophoresis at alkaline pH. On cation exchange HPLC HbX eluted after HbA_2 in the Hb C – window. Later on, DNA sequencing showed the presence of GAG → AAG substitution at codon 90 in the beta globin gene. This rare hemoglobin has been reported in some other populations and is called Hb – Agenogi. This is the first time this hemoglobin is reported in the Indian population.

Hb Hofu – (β^{126} (H_4) Val →Glu)

This sample showed an abnormal hemoglobin band (HbY) moving faster than Hb A at alkaline pH. On cation exchange HPLC ,Hb Y showed partial separation from Hb A in the form of a hump. DNA analysis later on showed a GTG → GAG mutation at codon126. This abnormal Hb (Hb Hofu) is reported in Indian and Japanese families.

Molecular characterization of δβ thalassemia and HPFH

This study was continued to look for the molecular mechanism leading to persistence of Hb F in adult life. Sixteen individuals showing raised Hb F phenotype were studied. DNA analysis identified HPFH – 3 deletion (48.5 kb) in 9 cases, the deletional inversion Gγ (Avδβ) ^{0} thalassemia in 7 cases.

A Gap PCR based approach was used to screen these individuals. Hematological and molecular data of these cases in given in the table.

	HPFH – 3 Mean (range)	**Deletional inversion Mean (range)**
RBC (10^6 / L)	3.24 (2.69 – 5.01)	4.83 (4.0 – 6.47)
Hb (g/dl)	12.3 (8.3 – 14.5)	11.3 (6.0 – 16.7)
MCV (fl)	80.1 (68 – 86.2)	69.3 (59.3 – 77.01)
MCH (p)	27.5 (19.3 – 30)	22.4 (16.0 – 28.3)
Hb A_2 %	2.6 (1.7 – 3.6)	2.4 (2.2 to 3.1)
Hb F %	35.2 (21 – 57)	20.1 (9.2 to 37.2)

During this period, we encountered a 32 years male, homozygous for the Asian Indian inversion $(A\gamma\delta\beta)^0$ thal, who clinically presented like a thalassemia intermedia with secondary hemochromatosis. Detailed hematological and molecular analysis of this case is given below.

RBC (10^6/L)	4.9	Sr Iron – 243 ug /dl
Hb (g/dl)	9.5	TIBC - 249 ug /dl
MCV (fl)	76.8	)% Saturation - 98%
MCH (Pg)	23.2	Sr. Ferritin 2406 ng /ul
Hb A_2 (%)	0.2	
Hb F (%)	99.0	
Mutation	Homozygous $G\gamma(A\nu\delta\beta)^0$ thalassemia	

The Effect Of Hydroxyurea On The Fetal Hemoglobin Response And Phenotypic Expression Of Sickle Cell Disease

Year of Commencement : 2004

Year of Completion : Ongoing

This project was sanctioned under the human genome programme and was initiated last year. As mentioned in the last year's annual report, 6 patients from the pediatric group who were followed up since the last 12 months and showed an increase in HbF without any episodes of crises after the commencement of hydroxyurea therapy, were followed up for another 12 months. Apart from hematological evaluation, alpha genotyping by multiplex PCR and Xmn1 polymorphism by restriction enzyme digestion were also done. They again showed a moderate increase in HbF without any episodes of crises and an increase in the Hb levels in 4 of the cases. Similarly, 3 adult patients who were followed up since the last 5 months were further followed up for 12 months and they also showed a similar pattern of increase in HbF and decreased episodes of crises after the commencement of hydroxyurea therapy.

Results:

Table 1: Results of the baseline data and data 24 months after treatment among the pediatric group

Sr. No.		PAT-IENT	AGE (years)	Hb Abnor-mality	Hb (g/dl)	RETIC (%)	F-cell (%)	Hb F (%)	Xmn I	Alpha-Genotype
1	Base line	N N	10	SS	6.1	9	61	15.2	+ / +	$-\alpha^{3.7}/\alpha\alpha$
	Follow up	N N			6.6	5	78	19.5		
2	Base line	J K	8	SS	7.5	13	44	11	+ / -	$-\alpha^{4.2}/\alpha\alpha$
	Follow up	J K			8.4	9	72	15.1		
3	Base line	R G	12	SS	9.5	3	67	16.4	+ / +	$-\alpha^{3.7}/\alpha\alpha$
	Follow up	R G			9.5	2	76	21		
4	Base line	K	13	SS	7.6	10	61	16.1	+ / +	$\alpha\alpha/\alpha\alpha$
	Follow up	K			10	7	82	20.6		
5	Base line	M K	11	S-Beta thal	7.2	5	75	34.7	+ / -	$\alpha\alpha/\alpha\alpha$
	Follow up	M K			9	3	85	41		
6	Base line	N M	8	SS	7.4	18	82	32	+ / +	$\alpha\alpha/\alpha\alpha$
	Follow up	N M			9.1	5	86	27.9		

Table 2 : Results of the baseline data and data 17 months after treatment among the adults

Sr. No.		Patient	Age (years)	Hb Abnor-mality	Hb (g/dl)	Retic (%)	F-cell (%)	Hb F (%)	Xmn I	Alfa – Thalassemia
1	Base line	S K	18	S-Beta thal	3.2	20	73	26.7	+ / +	αα/αα
	Follow up	S K			8.7	7	71	25.6		
2	Base line	K F	21	SS	9.5	10	78	20.9	+ / +	$-\alpha^{3.7}/\alpha\alpha$
	Follow up	K F			11.2	2	91	26.8		
3	Base line	R K	20	SS	9.6	10	79	24.9	+ / +	αα/αα
	Follow up	R K			11.7	2	90	26.9		

14 more patients from the pediatric group, 4 of whom had a history of Acute Chest Syndrome along with transfusion requirements and 10 suffered episodes of painful crises more than 5 times a year, were followed up since the last 6 months and showed an increase in HbF without any episodes of crises after the commencement of the treatment.

Table 3: Results of the baseline data and data 6 months after treatment among the pediatric group

Sr. No.		Patient	Age (years)	Hb Abnor-mality	Hb (g/dl)	Retic (%)	F-cell (%)	Hb F (%)	Xmn I	Alfa – Thalassemia
1	Base line	SW	15	S-Beta thal	9.3	5	50	16.1	+ / -	$-\alpha^{3.7}/\alpha\alpha$
	Follow up				10.6	2	70	30.1		
2	Base line	NV	6	SS	7.7	11	68	22.1	+ / +	$-\alpha^{4.2}/\alpha\alpha$
	Follow up				7.2	12	88	26.5		
3	Base line	SM	13	S-Beta thal	7.2	1	22	6.1	+ / -	$\alpha\alpha/\alpha\alpha$
	Follow up				8.3	12	39	12.5		
4	Base line	AS	18	SS	8.3	14	89	25.4	+ / +	$\alpha\alpha/\alpha\alpha$
	Follow up				10.9	4	95	30.8		
5	Base line	NA	8	SS	10.1	1	63	14.4	+ / +	
	Follow up				9.1	3	94	28.4		
6	Base line	RP	17	SS	10.6	3	53	15.1	+ / +	$\alpha\alpha/\alpha\alpha$
	Follow up				9.3	6	85	26.3		
7	Base line	AT	12	S-Beta thal	6.8	1	53	14.4	+ / -	$-\alpha^{3.7}/\alpha\alpha$
	Follow up				7.4	3	71	26.7		
8	Base line	SP	10	SS	9.2	3	63	10.8	+ / +	$-\alpha^{3.7}/-\alpha^{3.7}$
	Follow up				8	4	76	20.8		
9	Base line	AP	6	SS	7.7	3	71	23.2	+ / +	$-\alpha^{4.2}/\alpha\alpha$
	Follow up				9.2	2	83	24.2		
10	Base line	SB	11	SS	8.5	2	81	26.3	+ / +	$\alpha\alpha/\alpha\alpha$
	Follow up				8.9	7	87	27.3		
11	Base line	LW	9	SS	6.4	5	64	10.1	+ / +	$\alpha\alpha/\alpha\alpha$
	Follow up				7.3	11	80	21.5		
12	Base line	MD		SS	11.2	1	44	10.5	+ / +	$\alpha\alpha/\alpha\alpha$
	Follow up				11	4	89	26.8		
13	Base line	SJ	12	SS	4.2	6	80	27.5	+ / +	
	Follow up				12.8	2	92	31.9		
14	Base line	AN		SS	8.1	10	86	24.2	+ / +	$\alpha\alpha/\alpha\alpha$
	Follow up				12	10	94	27.5		

5 more patients from the adult group, all of whom suffered episodes of painful crises more than 5 times a year, were followed up since the last 6 months and showed an increase in HbF without any episodes of crises after the commencement of the treatment.

Table 4: Results of the baseline data and data 6 months after treatment among the adults

Sr. No.		Patient	Age (years)	Hb Abnor-mality	Hb (g/dl)	Retic (%)	F-cell (%)	Hb F (%)	Xmn I	Alfa – Thalassemia
1	Base line	KK	26	S-Beta thal	10	5	59	27.5	+ / +	αα/αα
	Follow up				9.1	4	65	27.5		
2	Base line	MP	22	SS	9	8	58	15.4	+ / +	$-\alpha^{3.7}/-\alpha^{3.7}$
	Follow up				11.5	5	75	19.3		
3	Base line	VS	18	S-Beta thal	9.6	5	46	15.5	+ / +	αα/αα
	Follow up				9.2	6	55	20		
4	Base line	KR	26	S-Beta thal	7	17	36	9.5	+ / +	αα/αα
	Follow up				7.3	6	53	16.3		
5	Base line	SS	32	S-Beta thal	6.8	4	35	10.3	+ / -	αα/αα
	Follow up				9.5	7	54	14		

During the HU treatment, the patient's hematologic status was monitored to rule-out falls in the neutrophil count to less than 2,500 per cubic millimeter, platelet count to less than 80,000 per cubic millimeter, hemoglobin of less than 6 g/dl. Also monitored were the hair loss, GI problem, rash and liver and kidney functions. One patient belonging to the pediatric group was withdrawn from the HU treatment due to reduction in the neutrophil count.

Hydroxyurea may benefit some of the thalassemia intermedia cases, however, the parameters influencing the response to HU have not been reported. In this study, α-globin gene status, β-globin genotype and the XmnI polymorphism, will be correlated with the HU response. 4 adult patients with thalassemia intermedia were selected based on their clinical condition and started on HU (500 mg per day). Their response clinically and hematologically will be evaluated over a period of 2 years.

α thalassemias are a heterogenous group of disorders and are characterized by impaired synthesis of α-globin chains resulting due to deletions involving one or both of the α-globin genes. The interaction of α thalassemia with HbS is known to influence hematological indices, reduce hemolytic rate and intra vascular sickling and increase deformability of the red cells. Decrease in HbS synthesis is due to decrease in the availability of α chains to combine with β^S chains. It is predicted that the lower concentration of HbS associated with α thalassemias would mitigate the clinical severity of sickle cell disease.

Out of 28 patients on HU treatment, 11 patients were found to have associated α thalassemia by multiplex PCR. Two patients showed two α-gene deletions and nine patients showed one α-gene deletion.

In-vitro cell culture studies will also be done where HU will be used at different concentrations to evaluate the F-cell response in the erythroid cell cultures. Hb analysis will be done by HPLC and F-cell distribution will be done by flow cytometry.