PEDIGREE ANALYSIS AND INVOLVEMENT OF PEROXIDASE IN SICKLE CELL DISEASE

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ABSTRACT

Routine hematological tests were performed in a family which was at risk for sickle cell disease. Cellulose acetate electrophoresis and Triton PAGE were employed to differentiate between various variants of hemoglobin. Based on the data a pedigree was constructed which indicated that few members of the respectively had received the S gene, some of them were sickle cell disease while few were sickle cell trait. Elevated levels of peroxidase enzyme in affected individuals reflect its involvement in RBCs destruction. Statistical analysis strengthen this statement (JPMA42: 186, 1992).

INTRODUCTION

All manifestations of sickle cell anaemia are due to the alteration in the molecular structure of hemoglobin[1] which is because of a mutation, resulting in a substitution of valine for glutamic acid residue at 6th position of the B-globin chain[2]. Anaemias and red cell senescence involve oxidative damage to the red cells suggesting that H2O2 is responsible for this damage both in senescent erythrocytes and in hemolytic reactions[3]. Sickled cells are more vulnerable to peroxidant threat[4,5] and accumulation of H2O2 can cause formation of monodialdehyde (MDA). This MDA can react with amino phospholipids of the membrane to produce novel lipid adducts that represent the membrane lipid cross linkage. In view, peroxidative membrane damage may result in the formation of irreversible sickle cells[6]. This study was undertaken to investigate the possible biochemical basis for sickling of red cells in sickle cell disease and trait and to trace defects in other members of the proband\'s family.

MATERIAL AND METHODS

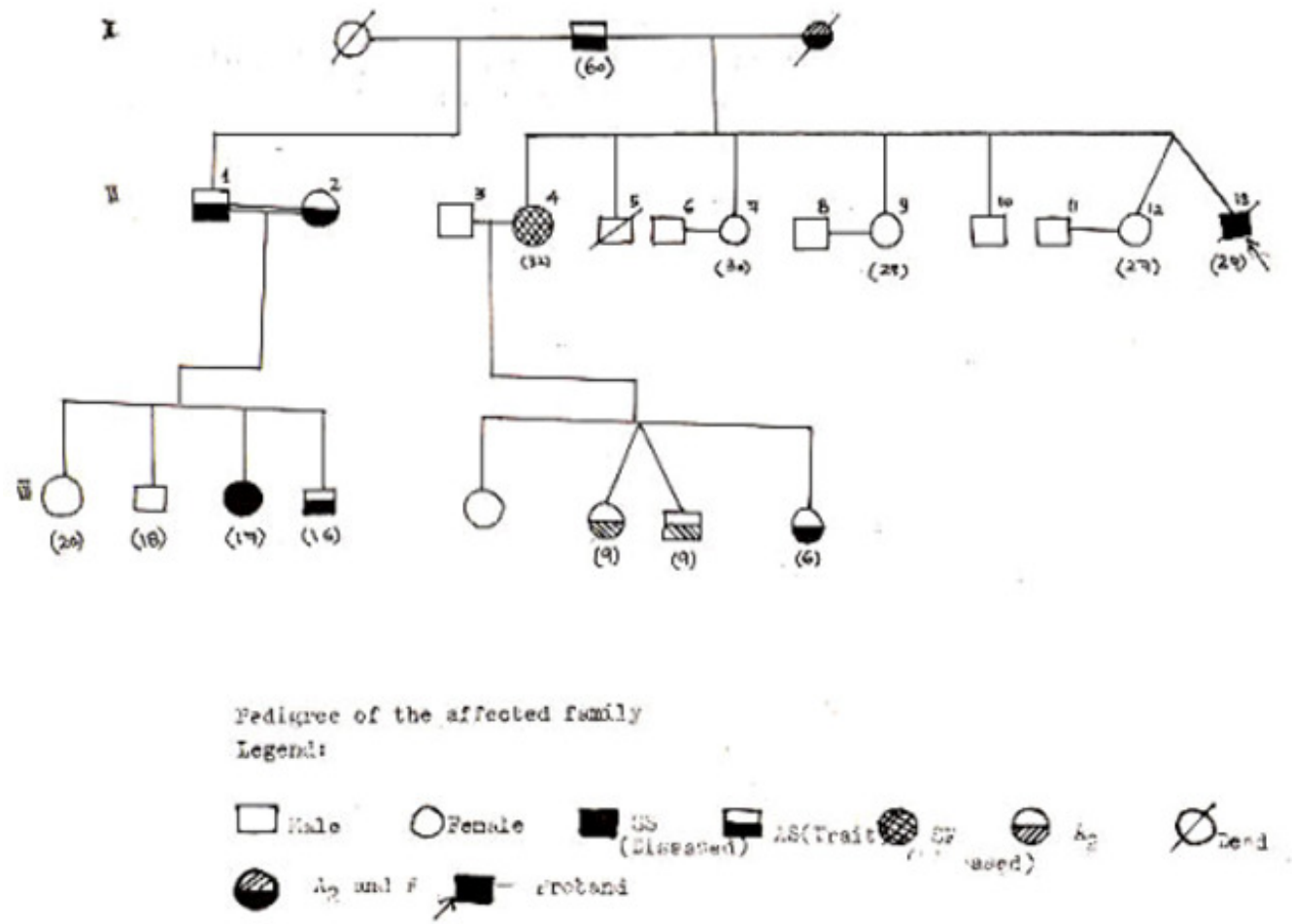

Figure 1. Pedigree of the affected family.

Figure 1 shows the pedigree of the family under study. 1113 was the proband suffering from sickle cell disease. Other members were then tested to trace the mutation in this family. Complete blood picture of different members of affected family was made by using Particle Counter model PC-604. These routine haematological studies include hemoglobin determination, PCV, MCV, MCII and MCHC. RBC morphology was studied using methylene blue and cosine. A 2% sodium metabisulilte solution was used for sickling test. In addition, cellulose acetate electrophoresis was done to study the percentage of different types. of Hb while Triton polyaerylamide gel eleetrophoresis (PAGE) was done for the detection of Globin chains in the manner described by Blanche et al[7]. The quantity of peroxidase was determined in plasma as described by Alvarez[8] whereas PAGE[9] was done for isoperoxidase separation using benzidine as the hydrogen donor[10].

RESULTS

Hematological investigation

TABLE I. Complete blood picture of members of affected family and controls.

Affected Family	Hb gms	RBC $x10^6 mm^3$	PVC	MCV u^3	MCH Pg	MCHC %
I2	9.6	3.4	29.0	85	28.0	33
II4	12.7	4.4	38.0	86	29.0	33
II10	14.5	5.1	45.0	88	28.0	32
II13	10.0	3.5	31.0	88	28.0	32
III4	13.6	4.7	41.0	87	29.0	33
III6	10.9	3.8	33.0	87	29.0	34
III7	10.4	3.6	32.0	89	29.0	32
III8	12.7	4.4	38.0	86	29.0	33
Controls						
1	10.5	4.8	35.0	92	27.6	30
2	15.8	5.0	42.6	92	34.4	37
3	15.8	6.0	42.6	92	34.4	37
4	9.1	5.4	31.3	75	21.9	29
5	18.0	6.0	35.0	80	25.0	33

Table I demonstrates the complete blood picture. RBC morphology of the members of affected family and control are shown in Table II.

TABLE II. RBC morphology and sickling test of members of affected family and controls.

Members of affected family	RBC morphology		Sickling test
I2	Hypocromia (+)	Anisocytosis (+)	Slightly positive
II4	Normocytic	Normochromia	Positive
II10	Normocytic	Normochromia	Negative
II13	Hypochromia (mild)	Anisocytosis	Positive
III3	Hypochromia	Anisocytosis	Positive
III4	Normocytic	Normochromia	Negative
III6	Anisocytosis (+)		Negative
III7	Anisocytosis (+)		Negative
III8	Normocytic	Normochromia	Negative
Controls			
1-20	Normocytic	Normochromia	Negative

H2, III and 1117 had hypochromia and anisoeytosis. Sickling test was positive in 4 members. The hemoglobin resistance to alkaline reagent was completely absent in III Maximum resistance was observed in 1113. In the control sample the range was 0.33-1.0% (Table III).

TABLE III. Percentage of alkaline resistance Hb in members of affected family and controls.

Members of affected family	Percentage of alkaline resistant Hb	Controls	Percentage of alkaline resistant Hb
I2	0.30	1	0.33
II4	2.00	2	0.33
II10	0.60	3	0.50
II13	2.88	4	0.50
III4	2.00	5	0.50
III6	0	6	0.33
III7	0.30	7	0.33
III8	1.00	8	0.50

Presence of Fibs was noted in 5 individuals. In II4 1113 and 1113 HbA was completely absent, instead they had elevated level of HbF. III4 and III8 exhibited the presence of HbS and HbA (Table IV).

TABLE IV. Different types of hemoglobin in members of affected family and controls (Sample 1).

Members of affected family	Percentage of HbA	Percentage of HbS	Percentage of HbF	Percentage of HbA 2/E
I2	65.03	34.97	0	0
II4	0	61.54	38.41	0
II10	100.0	0	0	0
II12	100.0	0	0	0
II13	0	75.69	20.83	3.48
III3	0	84.0	14.0	2.0
III4	63.64	36.36	0	0
III6	100.0	0	0	0
III7	94.08	0	0	0
III8	55.21	44.78	0	0
Controls				
1-20	100.0	0	0	0

Qualitative separation of Globin chains

When Triton PAGE was employed a disturbance in the position of bands (Rf-values) was observed in some members as compared to the control sample (Figure 2).

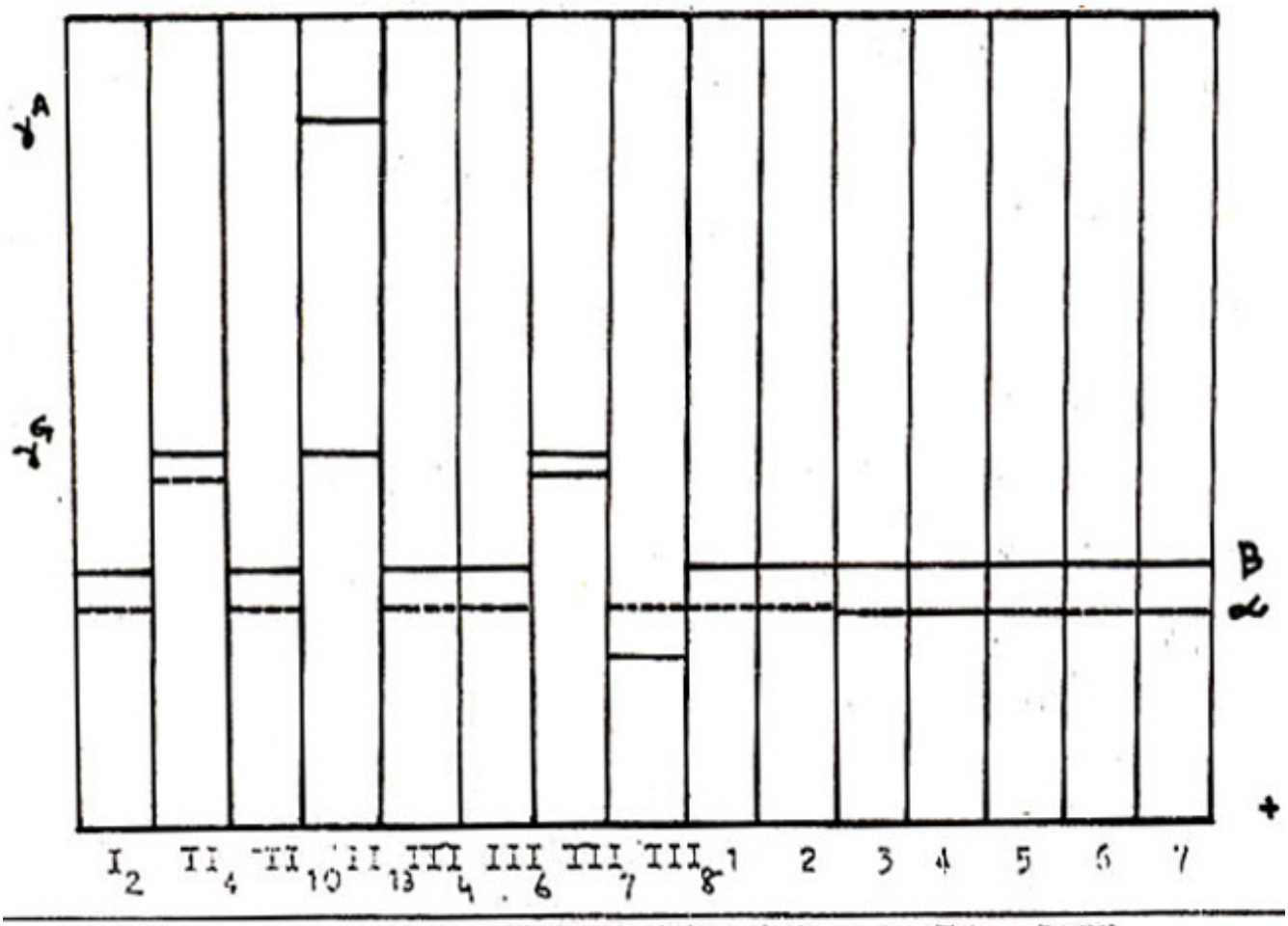

Figure 2. Zymogram showing globin chains using Triton PAGE.

Quantitative determination of peroxidase

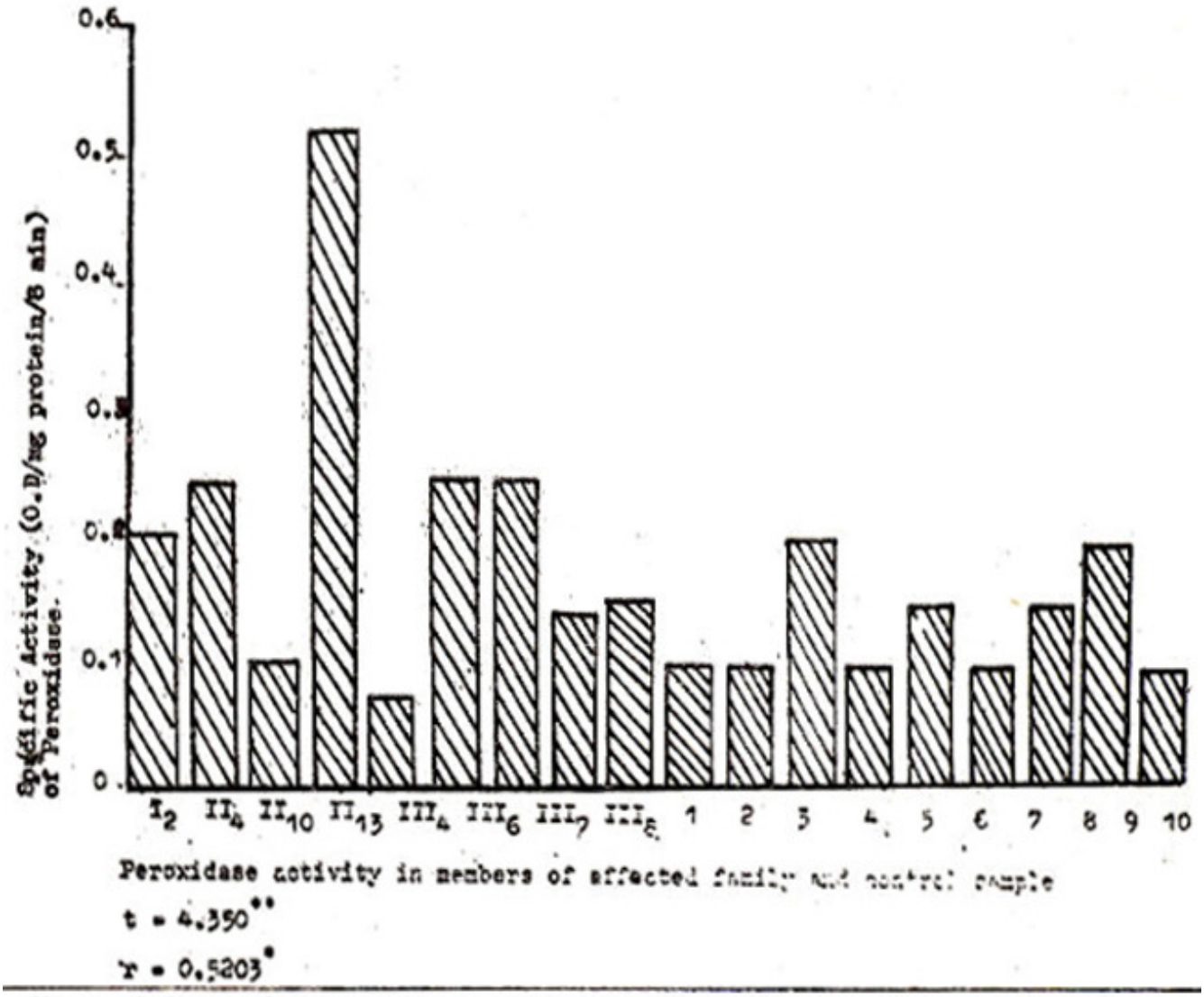

Figure 3. Peroxidase activity in members of affected family and control sample.

Figure 3 represents low peroxidase activity in III4 and higher levels in 1113 and 1113 as compared to control. When paired t- test was employed a significant difference was obtained in peroxidase activities of test and control samples. Moreover a positive correlation was also obtamed between HbS% and peroxidase activity[11].

Qualitative analysis of isoperoxidase

The banding profile explained in. the zymogram (Figure 4)

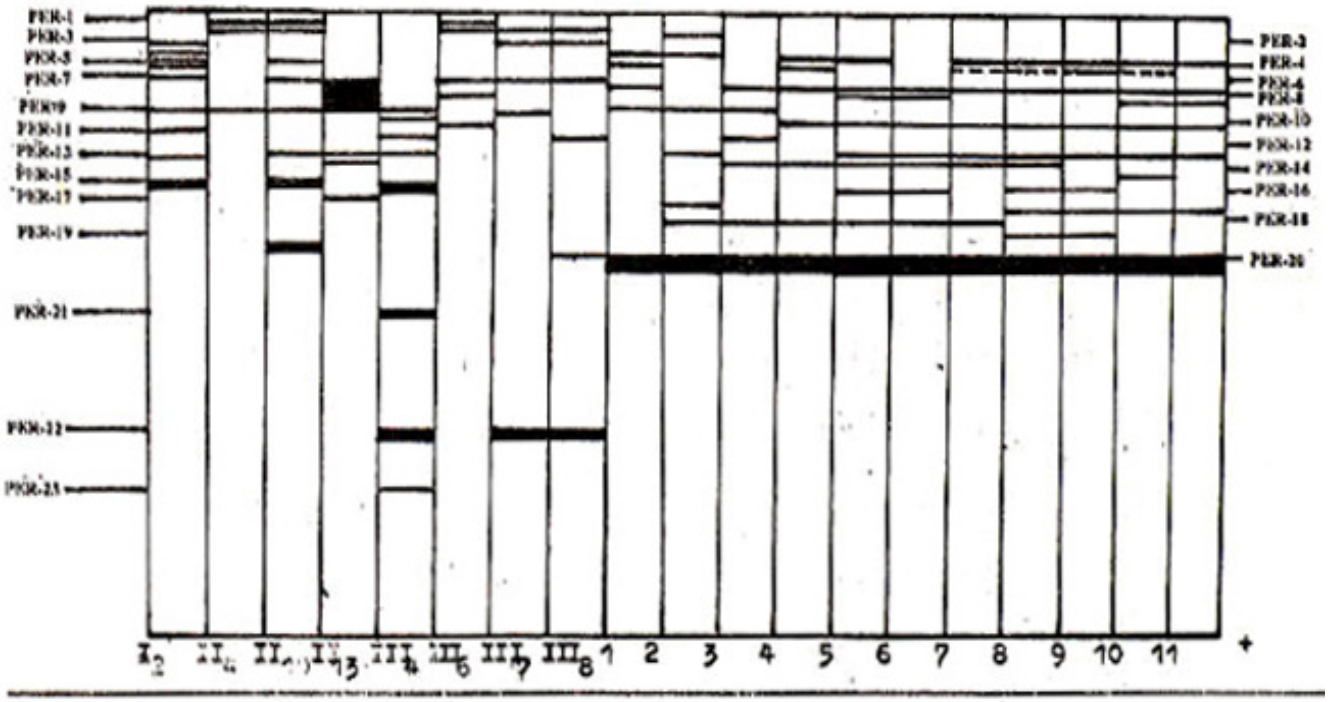

Figure 4. Zymogram showing isozymes of peroxidase in members of affected family and control.

indicates a variation in the number and position of isoperoxidases in some of the members of affected family as compared to control.

DISCUSSION

The complete blood picture of the proband family did not show a clear distinction from the control except that the concentration of Hb and RBC in I2 was slightly lower than normal. In II13 (proband) the amount of lib was at the lower margin of normal range. PCV, MCV, MCH and MC}IC arc the factors that have been proposed to influence the severity of sickle cell anaemia[12,13]. Results of the current work indicated that these factors were within the normal range in the members of affected family. However RBC morphology showed a variation in colour and size of red cells in four members. In I2 hypochromia and anisocytosis was observed. Mild hypochromia was noticed in II13 whereas anisocytosis was also present. Variation in shape of RBCs had also been noticed in 112, where most of the cells were moon- shaped in appearance and few cells had irregular margin. In 1116 and III7 variation in the size and shape had been observed. These results were consistent with the findings of Nash et al[14]. During current work based on RBC morphology sickling test was performed which was çositive in four members. Resistance of Hb to the alkali denaturation indicated higher levels in two members indicating the presence of high amount of HbF in these persons which was further confirmed by cellulose acetate electrophoresis. Krik[15] had used this test for the differentiation of Hb of a new born and patients with severe thalassaemia. Cellulose acetate electrophoresis of different members of the proband family indicated the higher levels of HbS and absence of HbA in 3 members, but they had increased levels of HbF. Noguchi and Schecter[16] reported that HbF replaced FIbS in the individual red cells to provide amelioration of clinical symptomatology. Higher levels of HbF also inhibit the polymerization of HbS and hence reduces intravascular sickling[17,18]. 114 although had higher level of HbS (61.54%) but simultaneously her red cells also contain HbF (38.41%); this could be the reason for

her to be asymptomatic. As reported by Boyer et al. [19] that expression oil cells seems to be independent of each other and those individuals who have HbF and HbS in every reticulocytes arc free of cell disease, it is supposed that severity of disease in any patient is influenced by the particular combination of alleles found at the modifier site. In the present investigation in III4 and III8 HbS percentage was 36.36% and 44.78% respectively but the sickling test was negative. Presence of HbA (63.64% and 55.21%) indicated that these persons had sickle cell trait. Allison[20] reported that red cells of asyinptomatic persons with sickle cell trait (A-S) require a lower partial pressure of oxygen for the production of sickling than do the red cells of sickle cell anaemia. In 12, 34.9% HbS/D and 65.03% HbA was observed, but the sickling test was slightly positive. Caweins et al[21] have reported mild sickling in S-D patient and HO sickling in A-S at all. Rate of sickling depends on the strength of interaction between HbS and other hemoglobins. HbD shows maximum interaction with HbS, due to this fact S-D patients do not have the manifestation of sickle cell anaemia. The presence of HbD led to the conclusion of his (I2) status as S-D syndrome. In 1117 HbA2 level was higher than control. He also had anisocytosis which indicates that he may be suffering from some hematological disease, probably thalassaemia trait but it has yet to he confirmed. 13 also had elevated levels of A2 (4.66%) and HbF (5.99%) probably 1117 had inherited the mutant gene from her grandmother. III6 was also negative for sickling although she had disturbance in her RBCs. She however had similar type of mutation as her brother (1117) and grand mother.

Triton PAGE

Results of Triton PAGE indicated the appearance of two bands in control as well as affected individuals. Band 1 (6.83 cm) and band 2(7.33 cm) present in five members of affected family were of a and chain respectively like control individuals. In case of II4 band 1 was present at 5.38cm and band 2 at 5.69 cm. By comparing the banding patterns of different globin chain reported by Blanche et al.7, it may be concluded that band 1 was of T6 whereas band 2 remained unidentified. Band I in 12, III4 and 1118 was analogous to the controls. This may be because these individuals had S.D Punjab and A-S and these mutations could not be identified by Triton gel.

Peroxidase analysis

Stock et al. [4] and Das and N air[5] have reported that sickle cells are more vulnerable to peroxidant threat. In the present research quantitative estimation of peroxidase indicated much higher levels in 1113 and 1113 than control whereas slightly higher levels were observed in II4 Significant differences in the quantity of pcroxidase between control and S mutation individuals and interdependence of HbS and peroxidase levels led to the conclusion that this enzyme plays an important role in the destruction of RBC cell membrane. These results are in agreement with the findings of Sushil and Shohet[6] who reported that accumulation of H2O2 can cause the peroxidation of membrane fatty acid and the formation of MDA which is the end product of peroxidase reaction. As sickle cells have a greater tendency to form MDA under minimal stress conditions; it may he suggested that the elevated levels of peroxidase in sickle cells resulted in autoxidation of unstable Hb and increased production of H2O2. Although M1) A quantity had riot been determined during present work, the higher levels of peroxidase in patients with sickle cell disease reflected the autoxida(ion of red cells. The quantity of peroxidase in I2 II I4 III6., 1117 and III8 was in the normal range because they were heterozygotes. In S-I) mutation either RBCs were resistant to peroxidative damage or had a higher degree of reversibility.

Qualitative estimation of peroxidase

The most frequent band was Px-8 among the members of affected family and control. Band 20 was present in control samples only; this band appeared only in H4 and H10. Per-22 was seen in 3 members of affected family while none of the controls had this band. These findings indicate that isoperoxidase pattern of persons with S mutation was different than controls. Based on the results, pedigree indicated that 12 and 13 had sickle cell trait (A-S). 12 had S-D Punjab. 13 had elevated levels of HbF she could have thalassaemia trait. Both parents li and 12 transmitted the S gene to two of their offspring, 114 and

1113, while II10 and H12 have inherited the normal version of the gene. From the second marriage I2 had only one son who got married to his paternal cousin and both of them were sickle trait CA-S) and hence transmitted the gene to their daughter 1113 who had sickle cell disease. 1114 and 1118 also had sickle cell trait. It is recommended that as this family is at risk for SS mutation the heterozygotes must not marry their cousins.

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